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Physiological Zoölogy

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PHYSIOLOGICAL ZOÖLOGY

Volume XXI JULY 1948 Number 3

ON THE MECHANISM OF ASTRAL CLEAVAGE[1]

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THE cleavage of marine eggs was one of the first loves of the early embryologists, and their successors in a slender but persistent series continue to give earnest thought to the problem of how a cell divides. One of the most spectacular of biological phenomena, cell division is the more difficult to analyze because of the elementary nature of the structures involved; its apparent simplicity captures the imagination and baffles it; the tremendous range of variations in this process challenges and, at the same time, discourages the investigator.

Among the various attempts to understand the mechanism of cell division have been some based on a likeness of the pattern of cleaving cells to other patterns which are found in nature, such as that of the lines of force in a magnetic field. Such theories, however, have gone as lightly as they have come, and it is only those based on extensive observation of dividing cells which compel serious consideration.

One of the earliest of these was the traction-fiber theory of Heidenhain, proposed in 1897, on the basis of long study of division figures in fixed material. Heidenhain represented the cleaving cell membrane by a steel band and the astral centers by small rings, from which rubber bands extended to the periphery as astral rays. The steel band was formed of two semicircles connected by hinged joints, so that, when the small rings were moved apart, greater tension was exerted on the periphery in the vicinity of the hinges and the frame was pulled inward until the circle was divided into two parts. This model, then, requires three assumptions, viz., strong attachment of the astral rays to the cell membrane, contractility of the astral rays, and autonomous elongation of the spindle. Heidenhain further argued on theoretical grounds that, if the model could be made with an elastic ring, more closely approximating the actual cell membrane, there would be found a stretching in the region of the furrow.

The surface-tension theory of cell division, as first advanced by Quincke, Rhumbler, and Bütschli and later sup-

[1] This paper is a continuation of *Physiological Zoology*'s informal series of synthesizing papers. It is based in part on work published in Japan during the war.

ported and extended by McClendon (1912), Robertson (1909), and Spek (1918*a*, *b*), is based on the observation that an oil drop immersed in water will divide into two parts upon the application of alkali, which causes changes in the surface tension of the oil-water interface, accompanied by streaming movements closely approximating those seen in various cleaving eggs. Superficially, the analogy is striking, but serious objections arise when its application is attempted. Moreover, there is a fundamental lack of agreement among the propounders of the theory: McClendon applies alkali at opposite sides of the drop, while Robertson applies it at the equator. Also it is obvious that the flowing movements within the oil drop are by-products of the movement of the interfacial film; in egg cells, on the other hand, a causative role in the cleavage process is attributed to these currents.

The astral-gelation theory was formulated by Gray (1924), supported by observations made by Chambers (1924). According to this theory, before cell division two elastic spheres, with or without rays, develop around centrosomes at the expense of fluid cytoplasm. As gelation proceeds and the surrounding fluid cytoplasm decreases in volume, the spheres come into contact in the median region, and eventually their centers are forced apart by the continued increase in their diameters, causing the observed elongation and, finally, cleavage. This theory finds support in many phenomena connected with cell cleavage. However, there are also a few well-founded facts which are irreconcilable with it. One of these concerns the measurements which have been made of cytoplasmic viscosity during the cleavage cycle, i.e., Heilbrunn (1928) (centrifuge), Seifriz (1920) (microdissection), and Carlson (1946) (Brownian movement) agree that, when the spindle is fully formed, the viscosity is low. There are also data obtained from the study of cell-surface behavior which cannot be explained by Gray's concept of simple gelation spheres. These data will be discussed below.

One other theory having many advocates is that which maintains that there is a superficial layer around the cell equator which contracts and pinches it into two parts. Most of the support for this idea comes from analogies with amoeboid movement (Chalkley, 1935), with gelation processes in inanimate systems (Lewis, 1939), or from a sort of common-sense approach to the necessity for answering a difficult question, together with the fact that there often are certain superficial layers in egg cells which can be invoked to dispel the great objection that there is no visible contracting band around dividing cells. Just (1939) supports this theory, citing changes in the relative thickness and behavior of the hyaline plasma layer at the time of cleavage.

In an effort to follow the changes in the cell surface which must be expected during cleavage, Katsuma Dan, T. Yanagita, and M. Sugiyama in 1935 developed a method of tracing the outlines of cleaving echinoderm eggs to which fine particles of kaolin were adhering. The linear distances between selected particles were measured and plotted against the stages in cleavage, and many such readings were compared, to give a dynamic picture of the activity of the entire cell surface during cytokinesis. The senior author has used these data, together with observations obtained by the use of other techniques, as well as certain facts already described in the extensive literature of the subject, to formulate a theory concerning the mechanism of cytokinesis. It is to be understood that this theory attempts to explain the division process

only in cells whose cleavage is accompanied by the appearance of a "typical" mitotic figure (i.e., a spindle with sufficient space for the development of asters) and an in-cutting cleavage furrow.

A study of the data obtained by the use of kaolin particles on cleaving sea-urchin (*Mespilia globulus*) eggs denuded of the hyaline plasma layer (Dan, Yanagita, and Sugiyama, 1937) showed that the curves representing the surface changes formed a continuous series, with the surface behavior at the spindle polar

FIG. 1.—Diagrammatic representation of the four regions in the echinoderm egg. *I*, polar region; *II*, subpolar region; *III*, subfurrow region; *IV*, furrow region.

regions clearly different from that in the equatorial region. For the sake of convenience in discussion, each potential blastomere was divided into four regions, named, respectively, "polar," "subpolar," "subfurrow," and "furrow" (Fig. 1). Particle distances located in any one of these regions showed approximately the same sort of behavior, but with slight differences peculiar to its region. For the polar, subpolar (Fig. 2), and subfurrow regions (Fig. 3) there was found consistently a gradual expansion of the surface with the onset of cell elongation; a maximum, which, however, was increasingly greater and correspondingly delayed as the furrow region was approached; and a falling-off from the maximum values in each case during the interkinetic period to a residual expansion which showed no further diminution. Only in the furrow region (Fig. 4) was there found a preliminary shrinkage, followed by a relatively tremendous expansion, which retained the characteristic, observed in the other regions, of reaching a maximum at the completion of cleavage and diminishing somewhat during interkinesis.

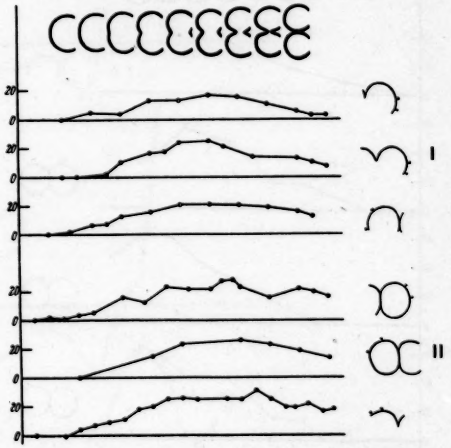

FIG. 2.—The upper half of the figure, designated as *I*, shows the behavior of the polar region and the lower half (*II*) that of the subpolar region. Each base line represents the value of the initial reading, while each mark on the left indicates a difference of 20 per cent. The position of the particles under observation is shown on the right. The points on the curves are distributed according to the scale drawn at the top of the figure representing successive stages of segmentation. The curves of *I* and *II* show a close similarity, although the curves of *II* remain always higher than those of *I*. It will also be noticed that the maxima of the curves of *II* are reached slightly later than those of *I*.

It was found that, when the hyaline plasma layer was left intact, these regional differences tended to be equalized, as though the outer layer was slipping over the true cell surface. A repetition of the experiment on the eggs of the sand dollar, *Astriclypeus manni* (Dan, Dan, and Yanagita, 1938), which has a very thin hyaline plasma layer, gave the anticipated result—there was much less differ-

ence between the curves with and without the hyaline plasma layer than there was in *Mespilia*, which has an unusually thick layer.

Since the first cleavage spindle in *Astriclypeus* lies perceptibly toward the animal pole, it is possible to distinguish

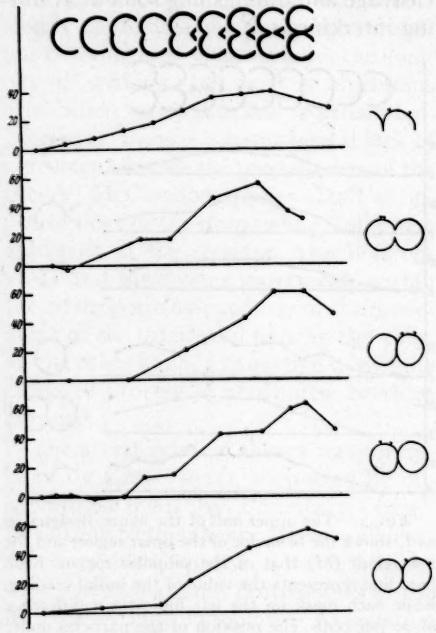

FIG. 3.—Percentage changes in particle distance in the subfurrow region. Note a gradual delay in the onset of stretching and attainment of the maxima. In these curves there is also a corresponding increase in the maximum values.

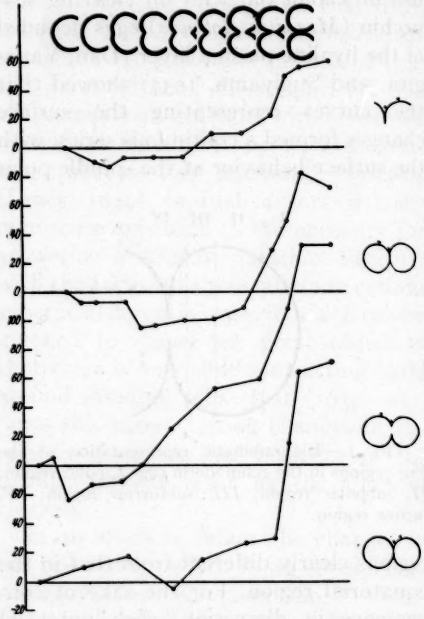

FIG. 4.—Percentage changes in particle distance in the furrow region. The initial shrinkage below the base line is a characteristic feature of this region. Also notice a great delay in the onset of stretching and corresponding delay in the attainment of the maxima.

the animal pole from the vegetal and the intermediate regions from either. Observations made on the basis of this distinction showed that the same preliminary shrinkage and subsequent great expansion are to be met with all around the egg in the equatorial region.

In order to observe the behavior of furrow surfaces which are unequally influenced by the spindle-aster system, particle distances were measured on dividing eggs of the snail, *Ilyanassa obsoleta*, which possess polar lobes immediately after the completion of cleavage (Dan and Dan, 1942). The curves for the furrow region (Fig. 5) followed the pattern of similar curves in echinoderm eggs, with an initial shrinkage of about 40 per cent, a later stretching to 250 per cent of the initial distance, and a final stretched condition, 190 per cent greater than the original surface. On the other hand, measurements involving the furrow which was formed between the vegetal side of one blastomere and the polar lobe attached to it (Fig. 6) showed no shrinkage but continuous stretching to about 500 per cent of the original value. This study led to two conclusions concerning the initial shrinkage in the furrow region: (1) Since it is found in eggs of

different phyla which also differ in cleavage pattern, it may be considered constant among, at least, egg cells cleaving with this general system of spindle, asters, and in-cutting cleavage furrow; (2) the presence of an underlying aster-

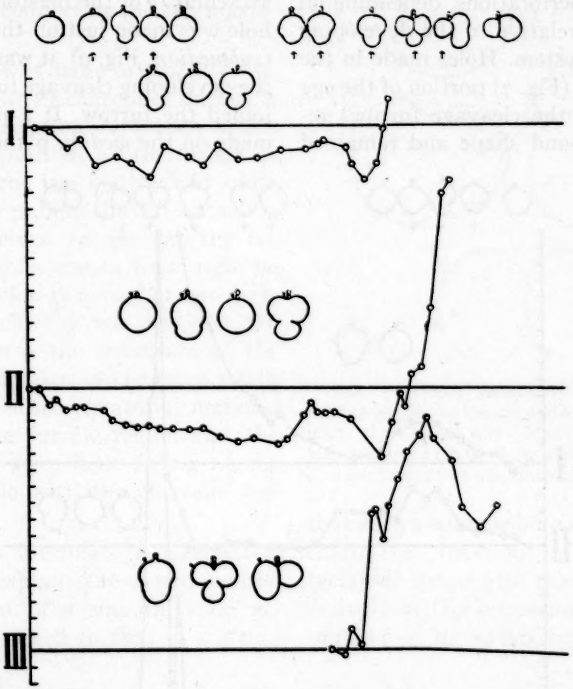

FIG. 5.—Surface behavior of animal-pole region of potential larger blastomeres in the eggs of *Ilyanassa*. Ordinates represent changes in particle distance, each division on the ordinates corresponding to 10 per cent difference. The phases of development in which readings are taken are shown by a series of drawings at the top of the figure. The specific position of the particle distance on the egg surface is illustrated by drawings accompanying the curves. Curves *I* and *II* show (1) the continuous shrinkage of this region during the lobe formation of the second maturation division and (2) the existence of a definite shrinkage phase prior to cell division. Curve *III* shows a great expansion during cleavage and a subsequent partial recovery, with a residual stretching. Because of a relatively wide spread of the particle distance in this case, the initial shrinkage phase is obscured.

spindle complex is necessary to cause its appearance, i.e., it occurs only in furrows which cut through the middle of a spindle.

In the course of attempts at microinjecting fertilized sea-urchin (*Pseudocentrotus depressus*) eggs (Dan, 1943*a*), it was found that, when the pipette pierced both surfaces of the flattened egg, a hole was often left after its withdrawal, which in favorable cases persisted even after the release of the egg from compression, so that it was possible to observe it throughout the ensuing cleavage. Using this as a second technique to establish a recognizable landmark on the surface of the egg, a series of such perforations was made in eggs in the stage at which the spindle system first appeared as a small dumbbell-shaped structure, clearly indicating the position of the future cleavage plane. Records were taken in the form of

camera lucida drawings showing the cell outline and the fate of the perforation throughout the subsequent cleavage.

Assembly of the data revealed that, as in the case of the adhering particles, there was a predictable difference in the behavior of the perforations, depending on their location relative to the developing aster-spindle system. Holes made in the extreme *lateral* (Fig. 7) portion of the egg (farthest from the cleavage furrow) retained their round shape and remained quite inert to the end of cleavage. Holes situated somewhat more medianly (*sublateral*, Fig. 8) were stretched out into a funnel or sort of groove in the blastomere surface, extending to the mid-point of the cleavage furrow (i.e., to the point of last attachment of the blastomeres). When a hole was made just off the median plane (*submedian*, Fig. 9), it was pulled toward the developing cleavage furrow, so that it joined the furrow. If a perforation was made on the *median* plane (Fig. 10), the

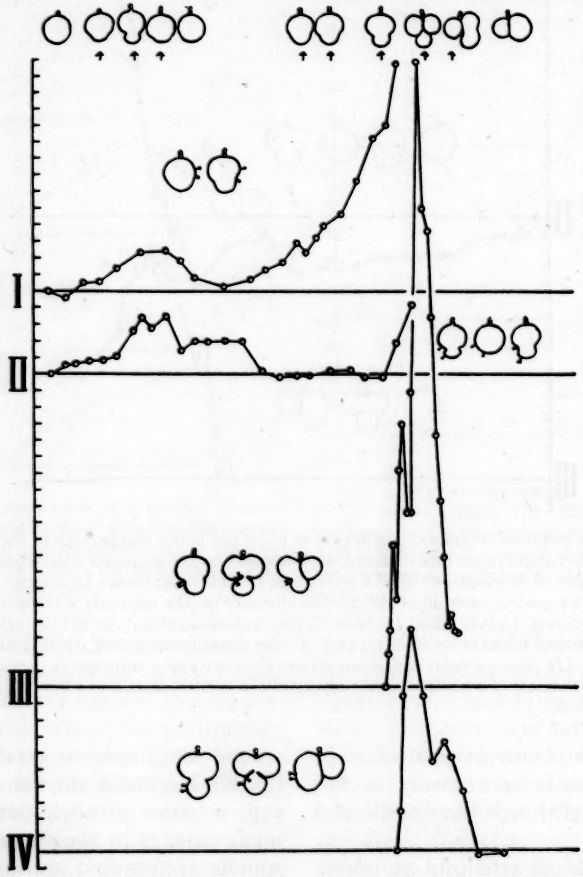

FIG. 6.—Surface behavior of the constriction between blastomere and polar lobe in the egg of *Ilyanassa*. Curves *I* and *II* illustrate that in this region there is no shrinkage accompanying the maturation division, but, instead, a distinct temporary stretching. Curve *III* shows the behavior of this region during cleavage. Curve *IV* gives, for comparison, an example of curves for the polar lobe.

side of the hole toward the spindle was pulled centrally to meet the cleavage furrow, which was advancing from the other side of the spindle, while the farther side of the hole remained inert, so that, at the completion of cleavage, a bridge of protoplasm was left connecting the two blastomeres.

An attempt was made to explain these results, using Gray's concept of the asters as spheres of gelating cytoplasm which would finally push each other apart as they used up the last of the fluid endoplasm, thereby pulling the cell surface in around themselves. In general, the behavior of the holes was such as might be expected of such a system, but two facts remained which did not fit into the scheme. One was the resistance of the bridge of protoplasm to the force which pulled all the other equatorial material away from the furrow region and the other was the invariable fusing of the submedian hole with the cleavage furrow.

In order to formulate a mechanism adequate to explain these two points, Gray's concept of a smooth, solid gel sphere was modified to that of a structure composed of spines radiating from a central core and inclosing fluid endoplasm in their interstices. It was believed

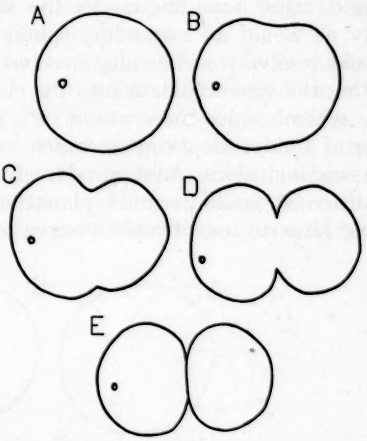

FIG. 7.—Serial camera lucida drawings of a perforation in the lateral region. The hole remains inert throughout the cleavage process. Room temperature, 15°o C. *A*, o minutes; *B*, 5 minutes; *C*, 9 minutes; *D*, 13 minutes; *E*, 20 minutes.

that such a spiny sphere would be able to fulfil the function of Gray's solid (gelated) spheres (of taking up the fluid endoplasm), by increasing the length and number of its spines and, incidentally,

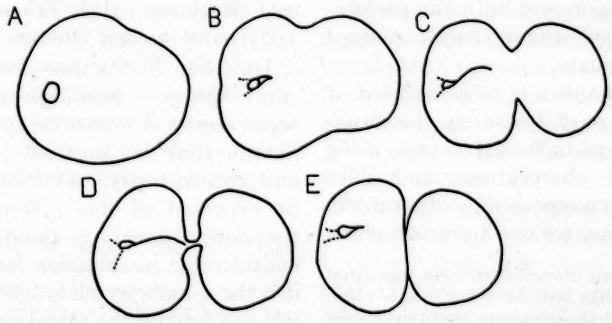

FIG. 8.—Camera lucida drawings showing the behavior of a perforation made in the central part of the sublateral region. Room temperature 13°o C. *A*, soon after the operation; *B*, after 4 minutes, showing the stretching of the hole (dotted lines show the contour of the cytoplasmic mass on the underside of the cell); *C*, 9 minutes, indicating a further stretching and groove formation; *D*, 16 minutes (the groove is almost cutting the blastomere into two parts); *E*, 41 minutes, interkinetic stage. The groove has disappeared, and the protoplasmic masses have coalesced. The hole remains in a stretched condition.

thereby providing more space to be filled with fluid. Pushing with many rays[2] against the cortical gelated layer, it would cause rounding-up in the same way as would an expanding sphere. It would resolve the difficulty involved in fitting the viscosity data into the cleavage scheme, since there would be a matrix of fluid endoplasm in which cytoplasmic inclusions could move freely. In addition, it would permit explanation of some hitherto inexplicable observations, notably the shrinkage of the surface in the furrow region and both the perforation results for which Gray's concept proved inadequate.

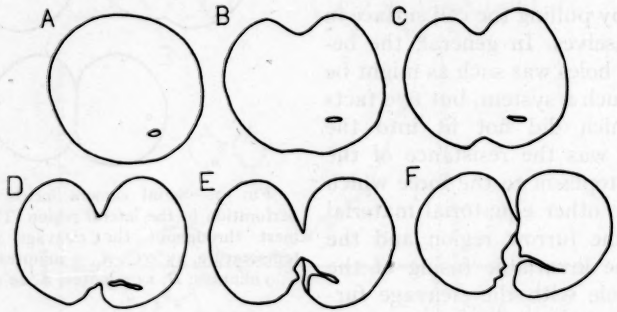

FIG. 9.—Camera lucida drawings showing the behavior of a submedian hole. Note that a normal furrow cuts in perpendicularly to the spindle axis, while the hole is stretched almost parallel to the spindle. The two join in later stages (*D–F*). Room temperature 13°.5 C. *A*, immediately after the operation; *B*, 4 minutes; *C*, 6 minutes; *D*, 9 minutes; *E*, 14 minutes; *F*, 21 minutes.

If this mechanism is to be utilized, it becomes necessary to clarify the situation with respect to the astral rays, using both original observations and data which have been reported by other workers. There is an extensive literature which establishes the fact that rays from the fully formed asters cross in the equatorial region. Briefly, this phenomenon has been described and figured in the following eggs: *Arbacia* (Wilson and Matthews, 1895); *Strongylocentrotus* (Danchakoff, 1916); *Toxopneustes* (Wilson, 1925); *Echinus* (Teichmann, 1903); *Chaetopterus* (Mead, 1898; and Lillie, 1906); *Polychoerus* (Gardiner, 1899); *Cycloporus* (Francotte, 1898); *Serpula* (Soulier, 1906); *Cerebratulus* (Yatsu, 1909); *Thalassema* (Griffin, 1899); *Piscicola* (Buchner, 1915); *Triton* (Rhumbler, 1903); and *Ascaris* (Boveri, 1888).

However, if it is thus proved that the spiny spheres commingle at the equatorial region, it immediately becomes apparent that no mutual pushing-apart and, consequently, no cell elongation can be expected of this system, and it is therefore necessary to assume, as there is considerable justification for doing, that it is the spindle which is actively elongating and forcing the astral centers apart.[3]

[2] This hypothesis disregards the idea that astral rays are not rodlike structures but simply channels of flow through the cytoplasm, since this concept does not seem to fit the observations made in the course of the present work and has not received extensive corroboration from other students of cytokinesis. If it proved necessary to provide for a flowing of endoplasm along the lines of the astral rays, a compromise could still be effected by postulating a tubular structure of the rays.

[3] Offsetting the tactical superiority which Gray maintains in requiring only one assumption (astral gelation) to explain a process for which Dan requires two (astral ray gelation and spindle elongation) is the fact that Gray's description contains a serious flaw with respect to the

Another pertinent observation which has been made by many investigators who have studied dividing egg cells is that of the so-called "fountain figure," formed by the rays at the polar extremes of the astral system (Wilson, 1925, *Toxopneustes;* Yatsu, 1909, *Cerebratulus*). Early in the cell-elongation process the astral rays can be seen to be curving away from the line of the spindle axis, as is shown in Yatsu's figures of the cleavage pattern in *Cerebratulus* eggs (Fig. 11). Dan interprets this as an expression of the yielding of the polar rays under the strain to which they are subjected between the elongating spindle and the resisting cell wall. If this is actually the case, the cortex against which these rays are pushing would be expected to spread, and this expectation is confirmed by the data from kaolin experiments (Fig. 2). It is further obvious that at this stage spindle elongation should proceed faster than cell elongation, and this is also ound to be the case.

The elongation of the spindle, pushing apart two spiny asters which hold in their interstices the fluid endoplasm, is an adequate system to serve as a substitute for the gelated-sphere mechanism. However, in order to bring the picture into line with more of the observed facts, it is necessary to include one more assumption, i.e., that the astral rays are firmly anchored in the cortical gel. It is certain that at least some of the rays can be seen extending to the cell periphery; the rays must be similar to the cortical gel in nature; it seems reasonably justifiable to include this assumption as part of the working hypothesis, in the absence of a method for making exact tests.

Given, then, the three assumptions that (1) the asters are radiate spheres, (2) the astral rays are anchored in the cortex, and (3) the spindle elongates autonomously, and using the observation tion that (4) the rays of opposing asters are mutually crossing in the equatorial plane, it was possible to construct a

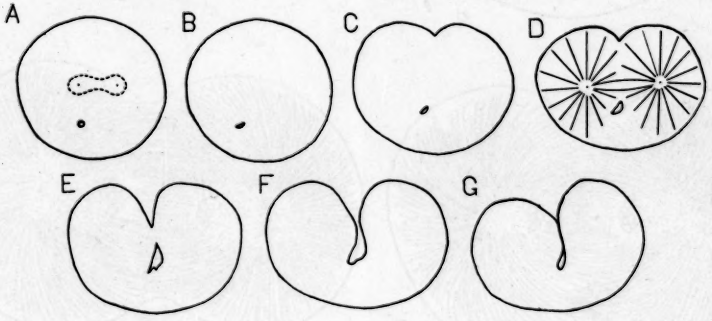

FIG. 10.—Successive camera lucida drawings of a perforation just off the median line. Note that the perforation curves slightly around the contour of the astral sphere to reach the mid-point of the spindle. Room temperature, 14°o C. *A*, immediately after the operation (a dumbbell-shaped patch indicating the karyokinetic figure is shown); *B*, 5 minutes; *C*, 7 minutes; *D*, 10 minutes (astral rays are semidiagrammatically represented); *E*, 13 minutes; *F*, 17 minutes; *G*, 29 minutes (interkinetic stage).

time relations of the process. He states that in the cleavage of *Echinus esculentus:* "As soon as the two daughter nuclei are formed, practically the whole of the cytoplasm . . . is occupied by the two asters whose rays extend almost to the periphery of the cytoplasm. So far, the cell has retained its spherical outline, but at this moment . . . the polar elongation begins" (1931, p. 196). According to Gray's scheme, by the time all the cytoplasm is filled by the asters, cleavage ought to be complete.

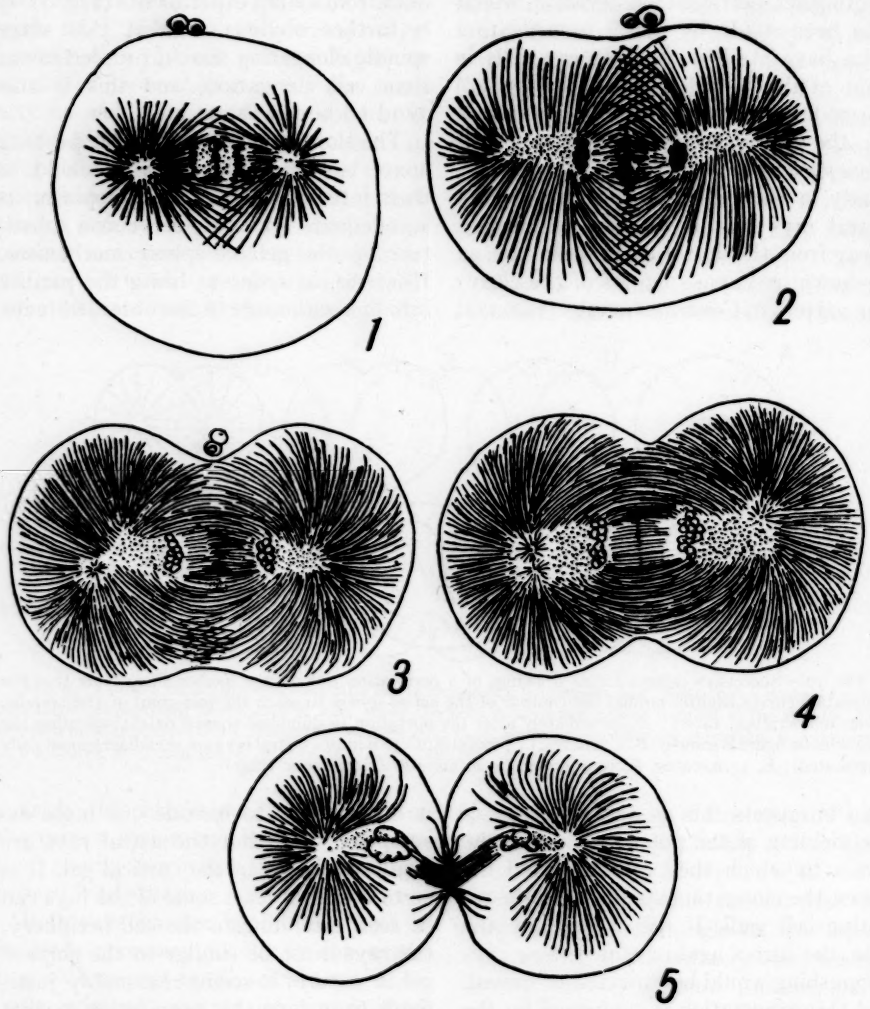

FIG. 11.—Reproduction of Yatsu's text figure *C*. Legends from Yatsu: "Five stages of the first cleavage, ×400. 1. Anaphase of the first cleavage mitosis. Crossing of rays, and the position of the spindle. Difference in distance between two centrioles at either end; 2. Telophase of the first cleavage mitosis. The centrosome has enlarged and been bent downwards. The crossing of rays has begun to loosen. A faint indication of fountain figure is seen at the pole region; 3. Telophase; a vertical section through the poles and a centriole. Constriction has begun on both the animal and vegetative sides. Sheath spindle has been formed. Fountain figures at the poles of the spindle have become more distinct; 4. Telophase (about the same stage as 3), a horizontal section through three centrioles. Sheath spindle is very well formed. Centrosome has greatly enlarged. The distance between the rows of karoymeres is approximately the same as the original length of the spindle; 5. Late telophase (a vertical section). Sheath rays have been formed. Fountain figure in the equatorial rays."

simple model in one plane (Fig. 12), consisting of wooden disks from which flexible wooden rays project, with an encircling rubber band tied to the tips of the rays. The spindle is represented by two overlapping strips of wood perforated at intervals, so that their combined length can be adjusted at will and held by a pin. Then, when the spindle length is increased, the shrinkage of the cell membrane at the furrow region is represented quite satisfactorily. However, plotting of the loci of a set of imaginary crossing rays gives the result shown in Figure 13, and it is immediately apparent that this is not a true picture of the situation at the completion of cleavage. Dan therefore introduced the further assumption, which is again in concordance with such evidence as there is, that (5) the crossing rays pull loose from the equatorial cortex as their asters are pushed apart. It is then suggested that the following process is at work:

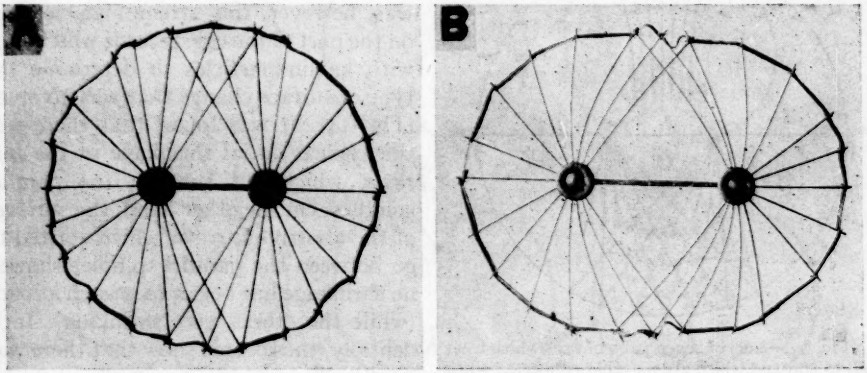

FIG. 12.—Model of centrally located karyokinetic figure, imitating the initial shrinkage phase. Astral centers are represented by wooden wheels in which bamboo rods are firmly implanted (imitating astral rays), which, in turn, are tied to a rubber band simulating the cell membrane. A wooden bar which represents the spindle is also firmly fixed to each wheel, the bars from the two wheels overlapping in the spherical condition (*A*). The bars have small holes along their lengths, so that they can be fixed in any desired length by putting a pin through these holes (*B*). In *B*, note (1) the flattening of the furrow regions, (2) the shrinkage of the furrow surface, (3) fountain-figured bending of the polar rays, and (4) consequent stretching of the polar surface. Note also that (5) the spindle remains straight after the elongation.

As the elongating spindle pushes the astral spheres apart, the brush of polar rays transmits the push to the lateral cortex, without, however, causing an equal amount of elongation of the cell diameter because the astral rays yield at first and bend slightly into the so-called "fountain-figure." The median rays have meanwhile been pulling at their cortical attachments, causing a shrinkage of their surface layers in the equatorial band which lies within the range of crossing. At this stage the astral rays are filling the cell, but, more than this, they are overlapping in the central region, so that here the fluid endoplasm is filling the interstices of a certain fraction of both spheres simultaneously. As spindle elongation becomes more extreme (the final length is about three times the maximum length in the spherical condition), the connection between the most-stretched crossing rays is strained to the breaking-point, and they are pulled loose from the cortex. As the two spheres are gradually pushed apart, they dissociate in the equatorial

region, and the endoplasm which they formerly shared now becomes insufficient to fill the two discrete spheres. Since there can be no increase in the volume of the system, (6) a suction force sufficient to pull in the equatorial cytoplasm and surface layers is developed; and, by the time that the spindle has ceased elongating, the equatorial endoplasm has all been sucked into one aster or the other, and cytokinesis is complete. This scheme may be variously tested as to its validity.

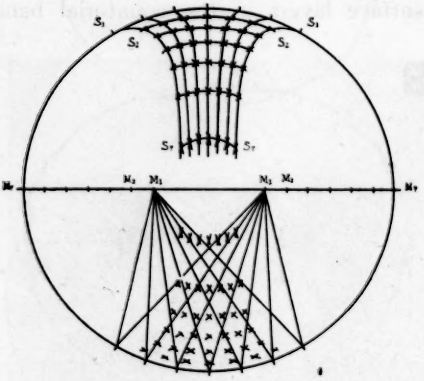

FIG. 13.—Loci of seven pairs of rays which meet end to end and which are movably connected when the spindle elongates. The lower half of the figure shows the positions of the tips of the rays for seven different lengths of the spindle, such as are indicated on the horizontal line. The upper half shows the same groups of points in terms of the surface. Notice that the rate of shrinkage of the surface is fast in the beginning and slow later, while the rate of the shift of the surface inward is slow at the beginning and fast toward the end. Note also that the wider the range of crossing of the rays, the greater the rate of shrinkage. The innermost position of the surface (S_7S_7) indicates that a complete division of a cell cannot be achieved by this mechanism alone.

1. *The initial shrinkage phase as a characteristic feature of furrows with underlying spindles.*—Besides the experiment discussed above on the eggs of the snail, *I. obsoleta*, another was performed to investigate the problem as it applies to sea-urchin eggs, using Wilson's (1901) technique for suppressing the first cleavage by treatment with 0.6 per cent ether on the eggs of *Strongylocentrotus* and of *Clypeaster*. In both these eggs, cytokinesis was suppressed at the first division; but at the second cleavage, unlike the *Toxopneustes* eggs used by Wilson, the binucleate cells divided into two binucleate blastomeres, with an abortive cleavage furrow forming between the asters without spindle connections. Since there was, however, this attempt at cleavage on the part of the egg, records were taken with kaolin particles to determine the type of surface change that was involved (Fig. 14). It was found that there was the typical initial shrinkage in the furrows, which cut through the parallel spindles. On the other hand, the surfaces of the abortive furrows which started to go between the parallel spindles showed no shrinkage but began to stretch at once (while the others were shrinking). Incidentally, the records show that there was practically no increase in the distance between the neighboring asters of the two aster-spindle systems as the asters increased in size.

2. *Simultaneity of the onset of spindle elongation, cell elongation, and initial shrinkage phase.*—The firm establishment of the simultaneity of onset of spindle elongation, cell elongation, and the initial shrinkage phase is essential evidence for the theory under discussion and gains further interest because it forms the crux of the argument between Gray (astral gelation) and Just (cortical contraction). In the present scheme, since the shrinkage of the presumptive furrow surface is an inseparable and immediate result of the spindle elongation, it must begin to appear simultaneously. The cell elongation, being buffered to some extent by the bending of the polar rays, may lag somewhat. Measurements were made with the same particle tech-

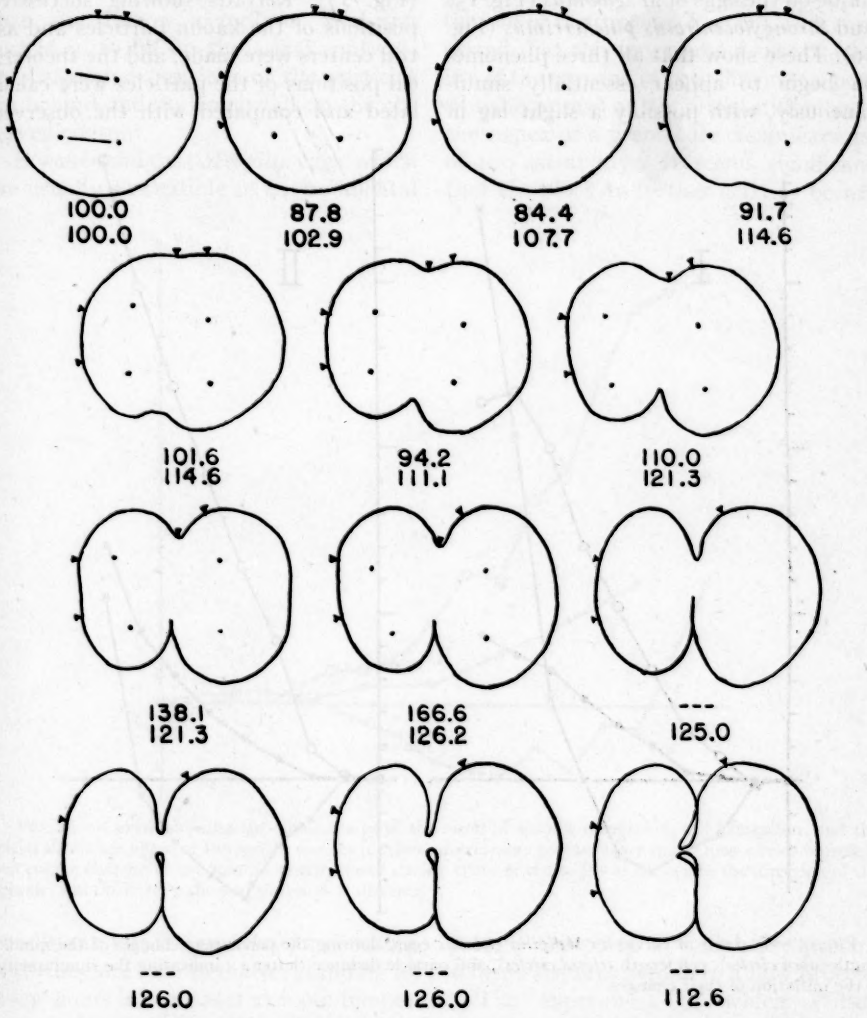

FIG. 14.—A series of camera lucida drawings showing the successive stages of cleavage of an ether-treated *Strongylocentrotus* egg (slightly compressed, in order to make the four astral centers come to lie in one plane). Four kaolin particles are also shown, two on the furrow which has an underlying spindle and two on the abortive furrow, lacking a spindle. The figures given with the drawings are percentage changes of respective particle distances. Note that the distance between two astral centers with a bridging spindle increases greatly and that an initial shrinkage of the furrow surface takes place, while the distance between centers of adjacent karyokinetic systems does not change noticeably and the furrow surface is stretched from the beginning.

nique on the eggs of *M. globulus* (Fig. 15) and *Strongylocentrotus pulcherrimus* (Fig. 16). These show that all three phenomena begin to appear essentially simultaneously, with possibly a slight lag in cell elongation in the case of *Mespilia* eggs.

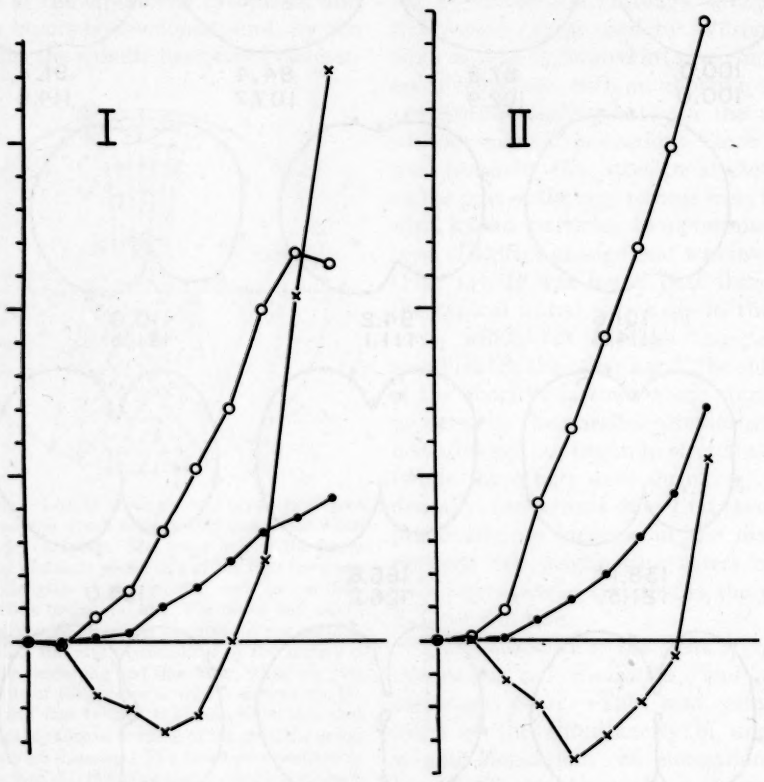

FIG. 15.—Two sets of curves for *Mespilia globulus* eggs, showing the percentage changes of the spindle length (*open circles*), cell length (*closed circles*), and particle distance (letter *x*), indicating the simultaneity of the initiation of their changes.

3. *Comparison of calculated shrinkage with observed shrinkage.*—Reference to Figure 13 will show that it is possible to calculate the amount of probable shrinkage of any segment of the furrow surface, if the assumption is allowed that a ray from each aster is attached to the cortex at each extreme of the given segment (Fig. 17). Records showing successive positions of the kaolin particles and astral centers were made, and the theoretical positions of the particles were calculated and compared with the observed positions. It is extremely interesting to find that, in the cases so treated, the calculated positions of the particles fall on the egg surface as long as this is shrinking—i.e., as long as, according to the present theory, the crossing rays are attached to the cortex and actively pulling on it. As soon, however, as the surface begins to be stretched (because the rays have finally been torn loose and the

asters have separated to such an extent that the furrow cytoplasm is being sucked in to fill up the astral spheres), the calculated positions of the particles lag behind and no longer fall on the observed contour.

It was found that *Mespilia* eggs, which are usually susceptible to environmental conditions, lose the capacity for cytokinesis if they are fertilized after standing for a few hours in sea water at room temperature (about 28° C.). The eggs complete mitosis, with considerable spindle elongation, and the beginning of a furrow appears; but the process uniformly stops at this point, and the cells round up again. Kaolin particles on such eggs and an analysis of the results by drawing intersection points as above (sec. 1) show that particle distances show only shrinkage and that the calculated intersection points fall exactly on the egg contour to the end of the process (Fig. 18). It is suggested that this represents a case of failure of the crossing rays to dissociate, possibly because of an abnormal stiffening of the cortex or a premature disappearance of the astral rays. It seems significant that the block to further activity occurs just at the point at which the present scheme requires a shift to a new process.

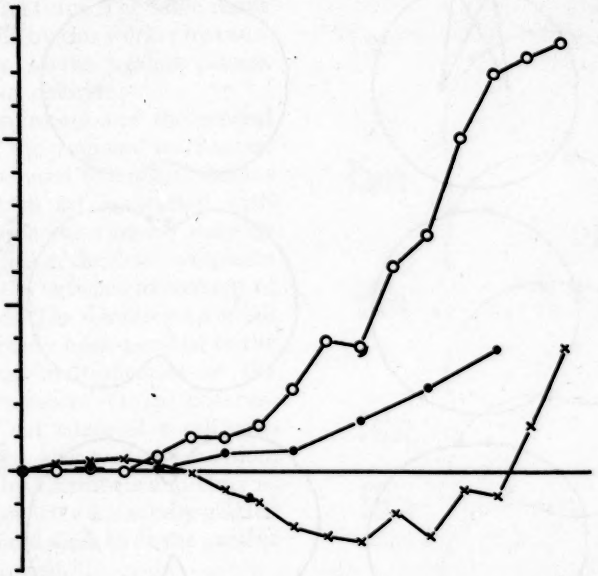

FIG. 16.—Curves showing the simultaneity of the onset of spindle elongation, cell elongation, and the initial shrinkage phase of the furrow surface in *Strongylocentrotus pulcherrimus* eggs. Open circles represent percentage changes of the spindle length, closed circles, those of the width of the cell in the direction of the spindle, and the letter *x* those of the particle distance.

The experiment in which Wilson (1901) found that treatment with ether prevented the development of asters and, consequently, of cytokinesis demonstrates clearly the role of astral rays in the cleavage of these large cells, in which it is necessary to manipulate a large amount of fluid with a minimum of gelation.

Other conditions which would result in the failure of cleavage would be those

which prevent either elongation of the spindle or departure from the spherical shape of the cell as a whole. In the first case, Frankston (1940) found that, after staining with neutral red, light stopped division of *Triturus* eggs in the metaphase. Hörstadius and Strömberg (1940) reported that carbon monoxide poisoning had an exactly similar effect. Finally, Beans and Evans (1940) found that cleavage of *Arbacia* eggs was arrested in late prophase, metaphase, or early anaphase by the action of colchicine.

The second condition—prevention of

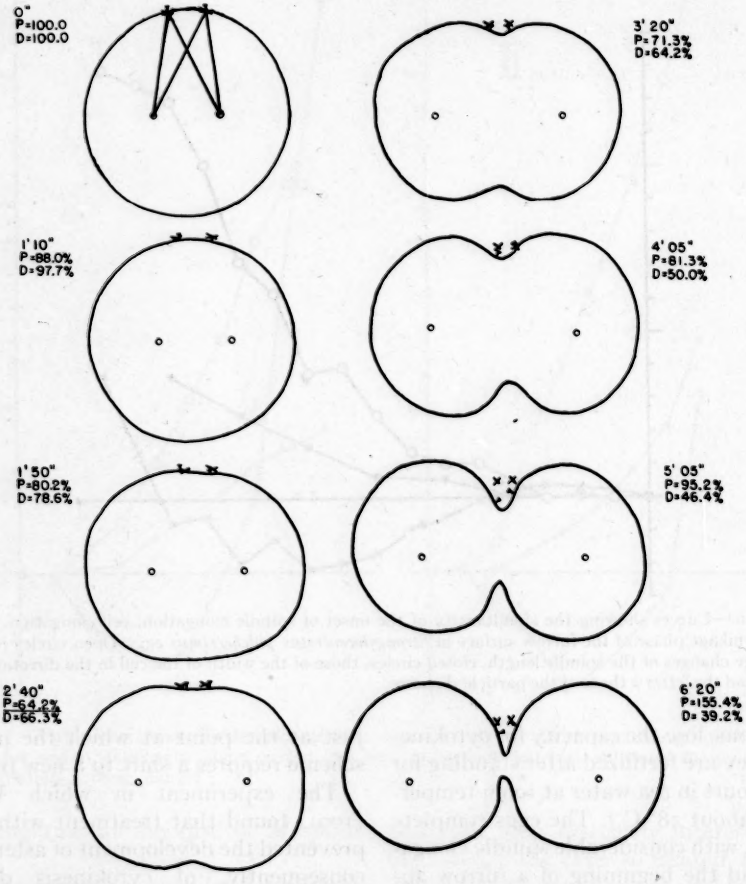

FIG. 17.—Successive camera lucida drawings of a cleaving *Strongylocentrotus* egg with the recorded positions of kaolin particles represented by solid triangles and astral centers by open circles. In the spherical condition (first drawing), two particles are connected to the astral centers by hypothetical rays. In successive drawings, by using the four lines connecting the particles and the astral centers in the first drawing as radii and taking the astral centers of successive stages as centers, two intersection points represented by letter x are obtained. Time of recording and percentage changes of the observed particle distance (P) and of the theoretical distance between the two intersection points obtained by drawing (D) are given. Note that (1) the initial shrinkage phase is quantitatively accounted for by the pull exerted by the rays and (2), during the shrinkage phase (down to the fourth drawing), the intersection points fall on the egg contour.

elongation of the cell—was produced by Sugawara (1943) by exposure of unfertilized *S. pulcherrimus* eggs to trypsin. In such eggs the perivitelline space is much restricted, and the fertilization membrane closely invests the cell surface. As a result, the cell is unable to elongate, and cytokinesis is entirely suppressed, the eggs developing into multinucleate, syncytia-like structures. The same result was also obtained by this worker by causing a hardening of the hyaline plasma layer with cerous chloride.

A further confirmation of the general applicability of the proposed mechanism is that it can be used to explain various phenomena which are associated with cleavage. *Endoplasmic currents* may be found to be eddies in the fluid cytoplasm resulting from the outward movement of the astral bodies. The *rounding-up of cell contours* has already been ascribed to the lengthening and multiplication of the astral rays. Chambers' (1919) observation, that the cut edges of a cell with asters retain the shape of the incision, has been used by Chambers and Gray as evidence that an aster is a solidly gelated body, but it would seem to fit the present scheme equally well.

Heilbrunn's *viscosity data*, as has been pointed out above, find satisfactory explanation in the proposed mechanism. *Recession of cleavage furrows under high hydrostatic pressure* Marsland (1938, 1939) and Pease (1940) could be explained as solation of the cortex alone, the presence of excess fluid cytoplasm preventing the creation of the suction effect. It is also possible that the solation would not stop at the cortex but would involve the asters and even the spindle. Cleavage furrows are also obliterated when dividing cells are placed in a *hypotonic medium*, presumably because of the increased supply of fluid. The *enhancement of cell elongation in a calcium-free medium* can be accounted for by supposing that the lowered resistance to elongation resulting from the softening or absence of the hyaline plasma layer results in less bending of the polar rays.

Chambers (1938) reported that when *one blastomere of a cleaving Arbacia egg is punctured in a calcium-free medium*, the furrow continues to advance for a short

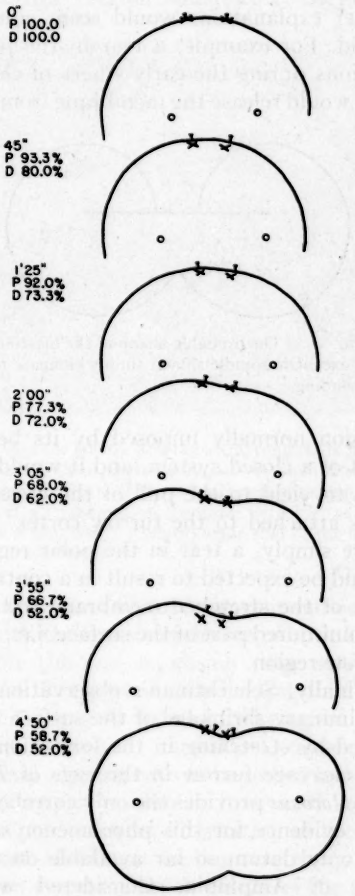

FIG. 18.—Analysis of an unsuccessful cleavage in *Mespilia*. The particle distance continues to shrink, and the intersection points remain on the furrow contour until the end of the process, showing the failure in the appearance of the suction mechanism.

time, in spite of the fact that the contents of the injured blastomere escape into the medium. He also found that, when the polar sides of both incipient blastomeres were punctured, the furrow wall apparently thickened and persisted after complete cytolysis had overtaken all the rest of the cell. These observations convinced him that the furrow wall is formed of especially thick cortex, but other explanations would seem equally valid. For example, a tear in the polar regions during the early stages of cleavage would release the membrane from the

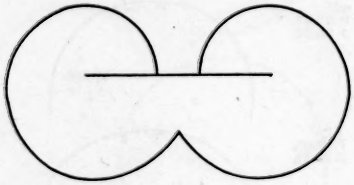

FIG. 19.—The probable shape of the blastomeres if an excentric spindle should simply elongate without bending.

tension normally imposed by its being part of a closed system, and it would be free to yield to the pull of the crossing rays attached to the furrow cortex. Or, more simply, a tear in the polar region would be expected to result in a contraction of the stretched membrane toward the uninjured part of the surface, i.e., the furrow region.

Finally, Schechtman's observation of preliminary shrinkage of the surface followed by stretching in the formation of the cleavage furrow in the eggs of *Triturus torosus* provides the only corroborative evidence for this phenomenon and the only datum so far available on the eggs of Amphibia. Considered with Rhumbler's (1903) figure of crossing astral rays in the egg of *Triton alpestris*, it offers hope that this scheme may be extended to include the cleavage of amphibian eggs.

Having achieved the formulation of a mechanism which can, with some success, answer the questions involved in typical cases, the next step is obviously to apply the concept to progressively more atypical examples of cell cleavage. As a beginning, the division of cells with excentric nuclei was examined (Dan and Dan, 1947*a*). This is a condition which occurs in many gradations; the egg of the sand dollar, *A. manni*, has its nucleus and subsequent mitotic spindle located off-center toward the animal pole, and the cleavage furrow appears first at this side of the cell. This egg is especially favorable material for such a study because the astral rays are particularly visible and well defined and can be seen crossing all along the median plane, with longer rays extending to the vegetal pole.

If the present mechanism is applied to such a system, the furrow would be expected to appear earliest in the region of the shortest rays, since these would be pulled in more for a given elongation of the spindle than would the longer rays and, after dissociation of the asters, a wider gap would be formed between the regions with shorter rays. However, if this situation is considered carefully, it is apparent that it would result in a cleavage pattern such as is diagrammed in Figure 19, which is an absurdity. Adaptation of the model illustrating regular cleavage to the condition of excentricity of the spindle gave a clue to the solution of the problem in the form taken by the extended spindle, which has automatically bent (Fig. 20) in response to the unequal pull exerted by the rays at the animal and vegetal poles.

Examination of *Astriclypeus* eggs determined that spindle bending is actually taking place, and it was found to be proportional to the degree of spindle excentricity (Fig. 21). There is a number of interesting points connected with the pat-

tern of the astral rays in these figures; for example, the clear areas free of rays which appear as the advancing furrow carries the formerly crossing rays inward to form the "sheath-rays" (see Yatsu, 1909, p. 387). Also in stages *2* (left-hand blastomere), *3*, and *4*, the bending of all the rays in unison lends the strongest support to the idea that there is a causal relation between the twisting of the asters and the bending of the spindle. In stage *6* it will be noticed that the rays on the vegetal side of the spindle have changed the direction of their bending, while a ray-free clear space has formed adjacent to the spindle remnant on the animal side. These are considered to be evidences indicating the beginning of a new formation of surface which will be discussed below.

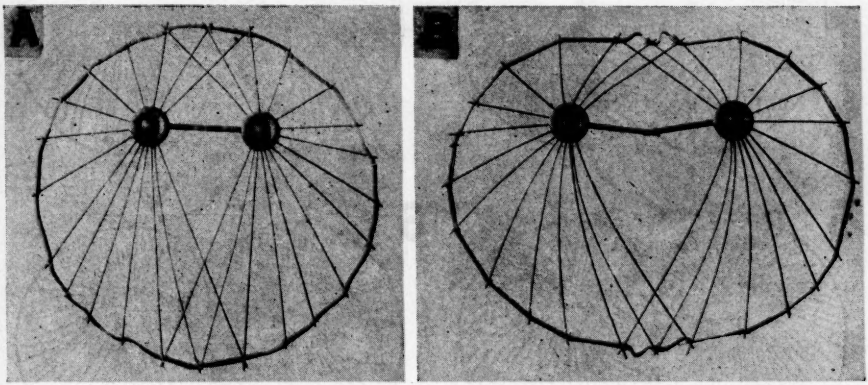

FIG. 20.—A model similar to that shown in Fig. 12, except that the spindle and the astral centers are made excentric (*A*). In *B*, after separating the astral centers and fixing their positions by putting a pin through the holes in the bars, we note the following points: (1) the fountain figure of the polar rays is obscured, and the majority of the rays turn in the same direction in a vortex configuration (compare with Fig. 12, *B*); (2) the innermost pair of the crossing rays at the upper pole is practically straight, while that of the lower pole is clearly bending; and, finally, (3) the spindle is flexed in the middle.

A study of these figures leads to the following proposition: *The cause of spindle bending is to be found in the one-sided pressure of the advancing animal polar furrow against the sheath rays which transmit it to the spindle.* As a corollary to this it is suggested that *the connection between the spindle and its asters is a relatively rigid one.*

Analysis of this form of cleavage by the kaolin method showed that, while the animal-furrow surface shrinks steadily and extensively and usually shows a degree of shrinkage equal to, or greater than, the calculated shrinkage, the vegetal-furrow region fluctuates between shrinkage and slight expansion and never attains the expected degree of shrinkage before the real expansion stage sets in. This is interpreted as indicating that the strain imposed by the twisting of the asters toward the animal pole causes the vegetal crossing rays to be pulled loose in an irregular fashion or in several steps, thus producing an alternation of the expected shrinkage and a stretching between the attachment points of the rays from the rotating asters (see Fig. 22).

In order to test the conclusion arrived at through the foregoing analysis—that the cause of spindle bending is an unbalanced condition of the mechanical strains

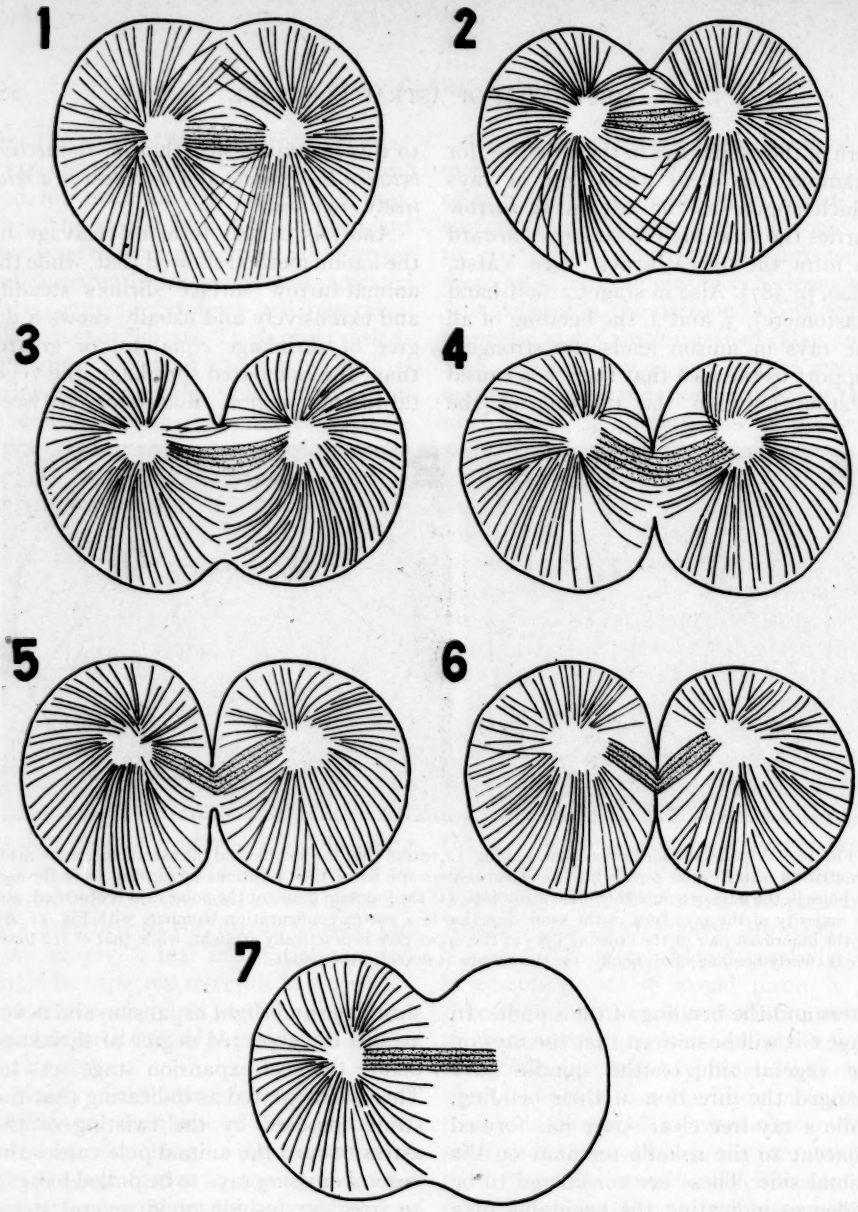

FIG. 21.—Tracings of microphotographs of dividing *Astriclypeus* eggs showing the changes of curvature of the astral rays in successive stages. The first drawing is made from a different egg, but the second to the sixth drawings belong to a series of photographs of a single egg. The spindle of this egg is more excentric than that of the first drawing. The seventh drawing is that of an egg with a far less excentric spindle. *1*, rays are practically straight. Theoretically the asters must have begun to rotate, but this cannot definitely be ascertained. *2*, note the appearance of the fan-shaped clear areas on both sides of the animal furrow in the expansion phase; sheath-ray formation, the bending of the vegetal crossing rays; the slight sign of spindle bending. *3*, further development of the fan-shaped bright portion, advance in the spindle bending and dissociation of the crossing rays of the vegetal side; also vortex configuration of the rays. *4*, approximate contact of the animal-furrow side with the spindle and the tendency of the rays of the animal side to return to a straight condition. Appearance of the fan-shaped area on the vegetal side. *5*, last moment of cleavage. Disappearance of the fan-shaped areas and definite bending of the spindle. *6*, interkinetic stage. Bending of the vegetal rays in the opposite direction (divergent bending). *7*, division of a cell with less excentric spindle. Notice usual fountain figure of the rays, instead of the vortex configuration, and apparent straightness of the spindle.

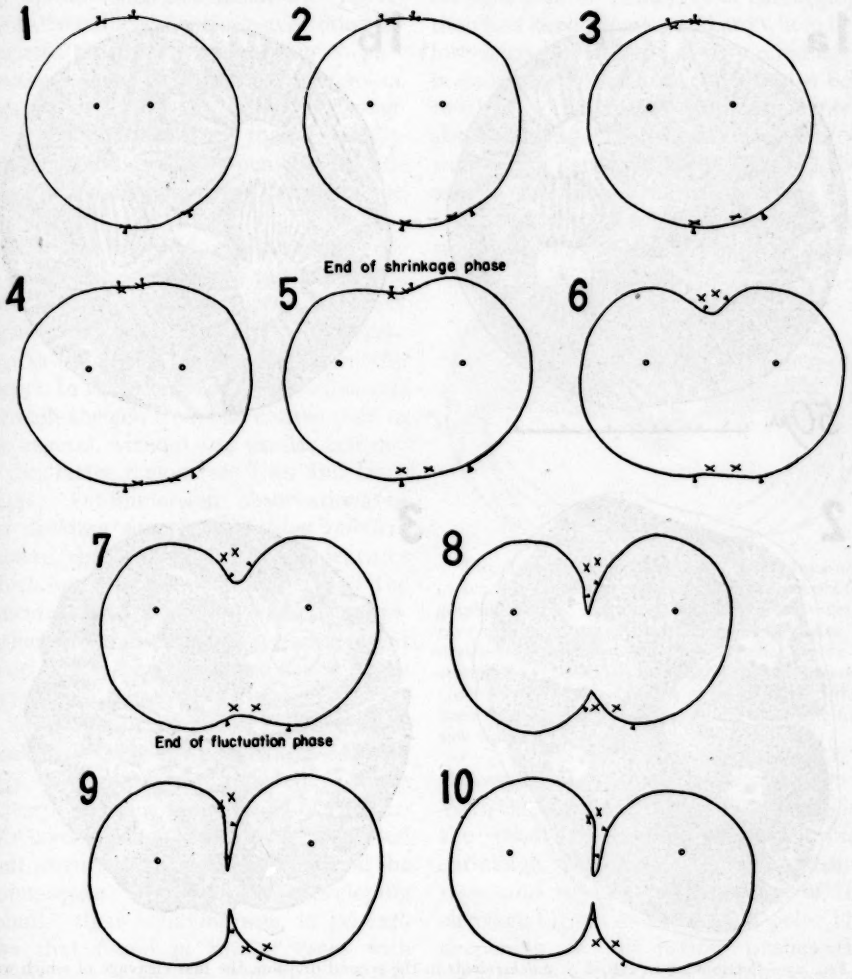

FIG. 22.—Another example of the geometrical analysis of an egg with extremely excentric spindle. The shrinkage stage of the animal pole lasts for five stages, and the fluctuation stage of the vegetal pole ends at the seventh stage. Note that the vegetal intersection points are sometimes within the contours.

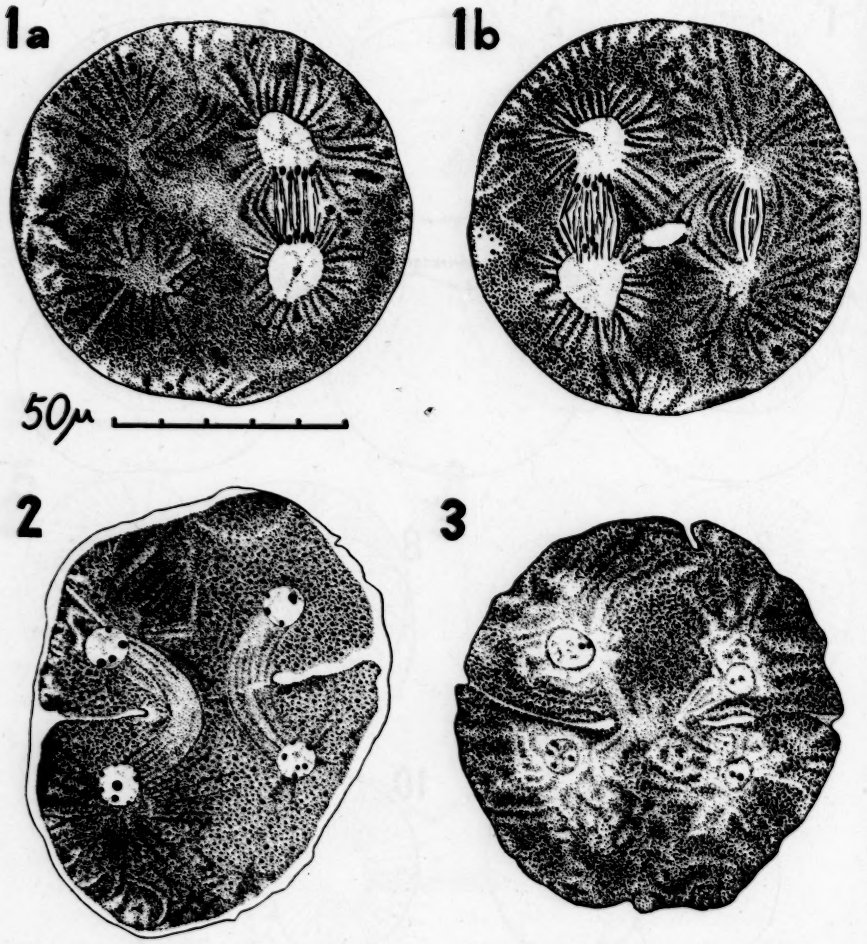

FIG. 23.—Sections of an egg of *S. pulcherrimus* in the second division, the first cleavage of which was suppressed by ether. Fixed in chrome-formal mixture, 1 *a* and 1 *b* are two successive sections of an egg showing two spindles and four asters; 2 is an egg cleaving into two binucleate cells, with bending of the spindles; 3 is another egg in a slightly later stage, with sharply bent spindles, the spindles being much more vague in this case.

acting on the animal and vegetal sides of the spindle—such a condition was experimentally induced by the use of ether to suppress the first cytokinesis in *S. pulcherrimus* eggs. Stained preparations of eggs fixed during the second cleavage (Fig. 23) amply show that normally straight spindles can be bent by the advance of a cleavage furrow from only one side.

After having performed experiments in which the spindle was pushed to the bottom of the cleavage furrow, the next logical step was to study coelenterate eggs in which this phenomenon normally occurs. In these eggs the furrow advances through the cell from the animal pole to the vegetal, without any similar activity in this latter region (see Dan and Dan, 1947*b*). Oil-immersion observations of the dividing egg of *Spirocodon saltatrix* showed that the two essential features which were found characteristic of the excentric mitotic pattern—i.e., astral rotation and spindle bending—are present to an even more pronounced degree in the cleavage of these eggs (Fig. 24).

Since kaolin particles could not be made to adhere to the surfaces of these eggs, perforation was resorted to in an attempt to trace the activity of the surface layer through cleavage. It was found that when perforations were made on the animal-pole side of the developing spindle, their behavior was, in general, like that found in similar cases with echinoderm eggs. On the other hand, holes on the vegetal side of the spindle, even though they were directly in the path of the approaching furrow, acted only as a block to further cleavage, instead of participating in the formation of the furrow as had been the case in echinoderm eggs. Moreover, it was found that holes even slightly off the median line in the vegetal region showed no form change whatever and often disintegrated before the completion of cleavage, indicating a greater instability of this region than had been encountered anywhere before, except in the case of sea-urchin eggs in calcium-free medium. It was also noticed that the surface scars which persisted after such holes had disintegrated were found decidedly shifted toward the animal-pole side of the blastomere.

By applying these data and observations, we may describe the "one-sided

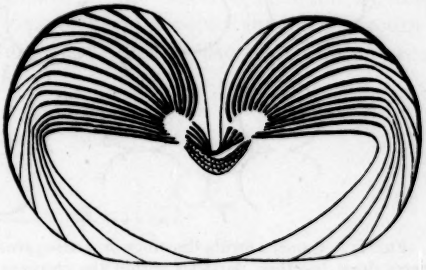

FIG. 24.—The condition of the astral rays and cortex of the medusan egg as interpreted from photographic records. The distal portions of the vegetal rays are greatly stretched. The animal cortex is drawn into the furrow, and the vegetal cortex is stretched to cover the lower half of the blastomeres. Greatly stretched parts are represented by thin lines. The ray-free area is spreading along the furrow wall.

cleavage" of medusan eggs as follows: With the beginning of spindle elongation, the usual mechanism causes surface shrinkage, dissociation of the crossing rays, and subsequent formation of the cleavage furrow at the animal pole. The deepening of the furrow pushes the sheath rays against the spindle, causing it to bend and the asters to rotate; and the fluidity of the vegetal region permits this condition to develop in an unbalanced fashion, so that the spindle finally takes a U-shape and the asters are rotated through nearly 90° from their original positions, while the vegetal materials are pulled away from the soft vegetal region around to the sides of the blastomeres. Toward the end of cleavage

there is a rounding-up of the blastomeres in this new condition, with the daughter nuclei and the spindle remnant situated well toward the vegetal pole.

The study of this medusan material was undertaken with the expectation that it would form the extreme case in the spindle excentricity series, but the result does not strictly fulfil that expectation. The true source of the aberrations in medusan egg behavior seems rather to lie in the difference in rigidity between the animal and the vegetal portions of the cell and, concomitantly, that of the astral system. This result was, of course, forecast by the fluctuating behavior which was observed in the case of *Astriclypeus* eggs with extremely excentric spindles and which, recurring in medusan eggs, seems to strengthen the conclusions which were drawn in that case, establishing a second graduated series, based on the degree of fluidity of the vegetal-pole area.

In the course of making observations on echinoderm eggs, a series of facts was brought to light concerning the behavior of the blastomere surface, particularly in the region of the completed furrow (see Dan, Dan, and Yanagita, 1938; and Dan and Dan, 1940). It is well known that, when echinoderm eggs cleave in sea water, the blastomeres, which are at first separated by the cleavage furrow, later during interkinesis are drawn together within the encircling hyaline plasma layer, so that they have a considerable extent of tightly apposed surface. In a calcium-free medium, this drawing-together of the blastomeres does not occur in the absence of the hyaline plasma layer, the daughter cells successively falling apart and continuing their division independent of one another.

Observation of kaolin particles which had been carried well into the cleavage furrows of *Astriclypeus* eggs showed that these particles were not trapped between

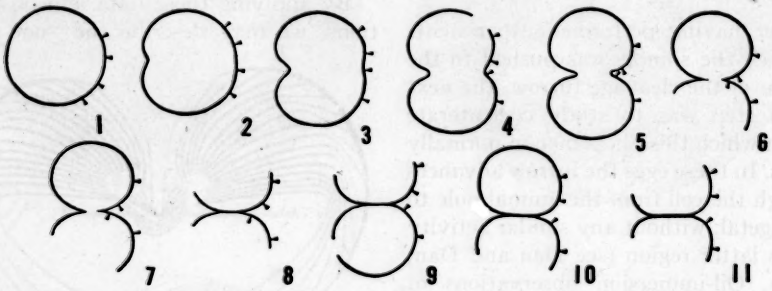

FIG. 25.—Camera lucida drawings of *Astriclypeus* egg cleaving in sea water. Note that, when the blastomeres draw together, particles within the cleavage furrow are not entrapped but are carried out of the furrow.

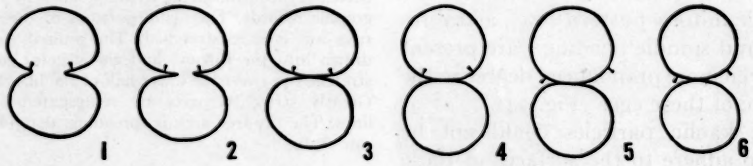

FIG. 26.—Successive drawings of *Astriclypeus* egg cleaving in calcium-free medium, showing that, despite the failure of the blastomeres to draw together, the particles are carried out of the furrow, just as they are when cleavage takes place in sea water.

the blastomeres when these became pressed together, as would have been expected, but, in the course of a few minutes after the completion of cleavage, were found on the blastomere surface well outside the cleavage furrow (Fig. 25). This was at first believed to be the effect of the hyaline layer which had not been removed in these experiments (in this form it is normally very thin and exerts a negligible effect on the recording of surface behavior). However, repetition of the experiment with eggs cleaving in a calcium-free medium gave the same results (Fig. 26): the particles were carried away from the point of last connection of the blastomeres during the early part of interkinesis. Distance measurements between the particles along the newly completed blastomere circumference gave the result shown in Figure 27 and led inevitably to the conclusion that there must be some sort of new formation of surface, presumably originating in this region where the blastomeres were finally separated from each other, to reduce the tension which had been set up by stretching the old egg surface to cover the two blastomeres. (The linear expansion of the surface in the largest optical section, which occurs when a sphere of a certain volume divides into two parts without changing the total volume, is 58.7 per cent over the original value. Measurement of drawings of cleaved eggs gave an increase in circumference of 54 per cent.) Measurements of drawings of eggs in interkinesis showed that the part of the blastomere surface on which particles were found in calcium-free medium or which was not included in the apposed surfaces in sea water is between 25 and 30 per cent longer than the initial circumference. Therefore, there remains an area

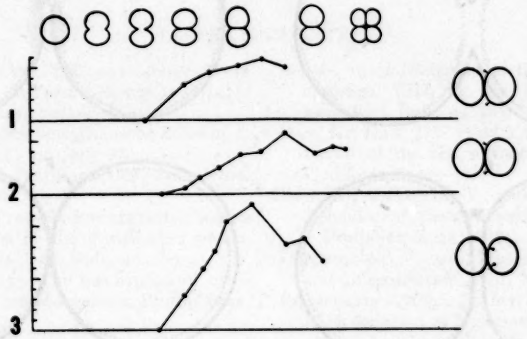

FIG. 27.—Graphical representation of the appearance of the particle-free area during interkinesis in calcium-free medium.

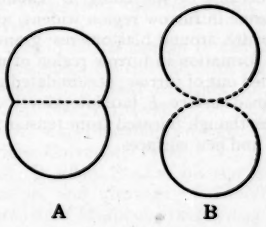

FIG. 28.—Diagrams of eggs at the middle of interkinesis in (*A*) sea water and (*B*) calcium-free medium. Broken lines indicate the newly formed surface, i.e., in *A*, the contact surface between the blastomeres; in *B*, the "particle-free area." Solid lines represent the old surface: in *A*, the "free surface of the blastomeres"; in *B*, the "part of the blastomere surface on which particles are found."

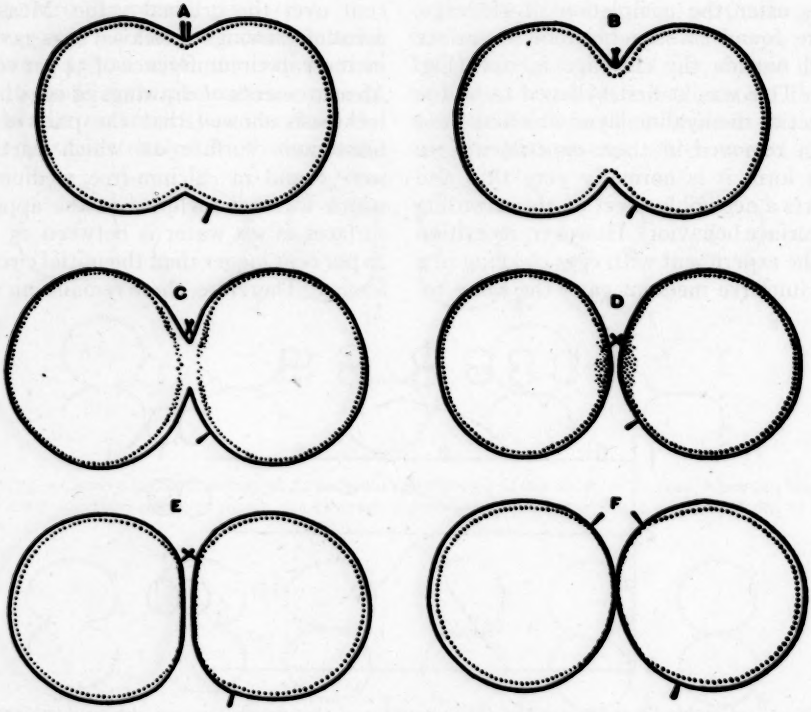

FIG. 29.—Diagram of typical *Strongylocentrotus* experiment to show, simultaneously, behavior of kaolin particles adhering to surface, extra-granular zone, and granular layer. (Changing outline of cleaving egg and positions of adhering particles are traced from photographs. Positions of granules are indicated by dots.) *A*, cleaving egg; kaolin particles are situated in furrow region; extra-granular zone has narrowed at poles, presumably because of stretching. *B*, kaolin particles are carried inward by deepening of furrow. *C*, extra-granular zone in furrow region widens, at poles becomes extremely narrow. *D*, extra-granular zone regains uniform width around blastomeres; granules are accumulated at point of last connection between blastomeres. *E*, formation in furrow region of new surface, lacking in extra-granular zone. Kaolin particles have been pushed out of furrow; accumulated granules have dispersed and are now lined up in close proximity to newly formed surface. *F*, late interkinetic stage; extra-granular zone over polar two-thirds of blastomeres has widened as though released from tension; upper particles are now well outside of furrow, at boundary between old and new surfaces.

represented by 25–30 per cent of the blastomere circumference which can be attributed only to a new formation of surface in the furrow region (Fig. 28).

In order to rule out absolutely the possibility that this result might be due to the presence of an extraneous layer or membrane slipping over the cell surface, it was obviously necessary to determine the behavior of the cell cortex itself in this connection. An opportunity to do this was offered by the observation of Motomura (1935), who followed the behavior of orange pigment granules in the cortex of *S. pulcherrimus* eggs by the use of a blue (Wratten No. 49) filter. Study of the eggs with this technique and also with strong white light under high power enabled simultaneous observation to be made both of the granule-containing cortex and of the particle-bearing surface. A typical experiment is presented diagrammatically in Figure 29. This is believed to be adequate evidence that there is a *de novo* formation of surface in the furrow region which occurs between the time of the completion of cleavage and about the middle of the interkinetic period.

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DIMINUTION OF RATE OF CELL DIVISION BY REVERSIBLE DYES COINCIDENT WITH ENHANCEMENT OF OXIDATIONS

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ONE of the problems confronting investigators of the physiology of cell division is the delineation of those metabolic processes current in the cell which contribute to division, as apart from those which transform energy for other purposes, such as maintenance of cell structure, in a physicochemical, as well as a morphological, sense. The latter would include, among others, the semipermeability of the plasma membrane, the potential difference between the inside and the outside of the cell, the differential distribution of ions between the environment and the cell interior. A survey of certain other aspects of the general

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problem of the functional correlatives of cellular metabolism has been made elsewhere (Shapiro, 1939).

Although it was originally postulated by Loeb (1913), from observations on the increase in oxidations in sea-urchin eggs on fertilization, that fertilization resulted in a general acceleration of oxidative processes, later investigators (Loeb and Wasteneys, 1912; Tang, 1931; Whitaker, 1931, 1933) demonstrated exceptions to this for the period immediately following activation. However, in both echinoderm (Warburg, 1931) and worm (Whitaker, 1933) eggs, irrespective of the direction of change after activation, the rate of oxygen uptake increases considerably during normal cleavage and differentiation in the period from several hours to a day after fertilization. This is true for a variety of temperatures.

Is there a general relation between enhanced oxidative activity and rate of cellular division? Does increase in the metabolic rate by any means whatsoever lead to energy production which is utilized directly in accelerated cell division? Reversible dyes like methylene blue, toluylene blue, brilliant cresyl blue (Barron and Hoffman, 1930), whose E'_0 (the electrode potential difference at pH 7.00 between the normal hydrogen electrode and an equimolecular mixture of oxidant and reductant) lies in the range +0.011–0.115 volt, are known to penetrate the cell and stain it readily (Harvey, 1941; Brooks, 1927*a*, *b*) and considerably elevate the oxygen uptake of marine eggs. The concentration required to produce an increase in rate of oxygen consumption of *Arbacia* eggs is very low, of the order of 10^{-4} M. Barron and Hamburger (1932) found that toluylene blue, 1.253 mM concentration, accelerated oxygen uptake in unfertilized *Nereis* and starfish eggs by factors of 5.65 and 2.56, respectively, whereas fertilized sea-urchin eggs were elevated 3.14 times. Brooks (1943) obtained an average increase of 12 per cent in oxygen uptake by fertilized *Arbacia* eggs in 0.002 per cent methylene blue, and Clowes and Krahl (1936) observed significant increases, at about 10^{-4} M methylene blue and upward. Taking into consideration the range of dye concentrations employed and the use of different dyes, it was felt that, although no direct measurements of oxygen uptake were made in the present experiments, the results of the three investigations cited above on the same cells (fertilized *A. punctulata* eggs) could safely be assumed to hold here, qualitatively at least, and that significant elevations of oxygen consumption were produced as compared with the controls.

EXPERIMENTAL PROCEDURE

The procedure was standardized and reproduced in each experiment as follows. The amount of dye used was very small (a fraction of a milligram); it was dissolved in 0.05 ml. distilled water; the drop was warmed carefully over a microflame to accelerate solution; and then 0.5 ml. of sea water was added. For the controls, 0.05 ml. of distilled water was added to 0.5 ml. of sea water. Eggs suspended in 2.5 ml. of sea water were added to both dye and control solutions, and then each was poured into a 300-ml. Erlenmeyer flask. The cells were thus in a shallow depth of sea water, facilitating gaseous interchange. The flasks were kept in a constant-temperature water bath, usually at 26°.1 C. ($\pm$0°.002) and, after temperature equilibration, inseminated at the same time by adding to each flask six drops of a previously prepared suspension of sperm in sea water. Both flasks were shaken gently while immersed, to distribute the sperm, and then clamped in stationary position in the thermostat, and moist oxygen passed

slowly over the sea water to insure adequate oxygenation. A partial pressure of 50 mm. of oxygen suffices (Tang and Gerard, 1932). The passage of oxygen also aided in preventing accumulation of carbon dioxide, which might by itself retard cleavage (Smith and Clowes, 1924). Samples of the eggs were removed in a pipette at regular intervals and fixed for counts of per cent of cleavage, according to a technique previously described (Shapiro, 1941). An alternative method of counting is to place the fixed eggs in a Syracuse watch glass on the stage of a microscope, attached to the movable stage. The microscope field is then scanned, as in a television raster, using a counter to record cell divisions. This enables the observer to study the cell structure under higher power and better definition than is possible with the dissecting microscope. The eggs used in each experiment were secured from a single urchin, in order to work with the most homogeneous cell population. Prior to using the eggs, a test fertilization was made to insure that a high percentage of membranes would be obtained.

When dye was added to the cell suspension, it tended to clump a portion of them. Microscopic examination revealed no visible damage, nor was subsequent cleavage of the cells prevented.

A note on the solution of methylene blue and the other dyes employed should be added here, since it is pertinent to interpretation of the results. Methylene blue is easily soluble in distilled water, but sparingly soluble in sea water. The technique generally adopted is to dissolve the dye in a small amount of distilled water and then mix this with sea water. That this procedure must be resorted to appears to indicate that the dye is brought into some kind of colloidal solution or minute dispersion by this technique; indeed, close examination of such a "solution" of methylene blue will often reveal fine particles. Hence to speak of "molar concentrations of the dye," as is done in this paper (and perhaps in investigations of others where sea-water solutions were used), is not strictly correct, since the dye does not appear to be entirely in true solution, and the concentration referred to merely indicates that a certain quantity of dye has been added to unit sea-water volume. It is conceivable that the particle size may vary as a function of the method of preparation and also as a function of time. The spread of the points shown in Figure 3 may be due in part to this difficulty in uniformly dissolving the dye in sea water and in part to intrinsic variability in response of the eggs of different animals. The amount of water added was insufficient to render the solutions sufficiently hypotonic to retard cleavage on that account alone. The resultant concentration of the sea water used in these experiments was approximately 98.5 per cent, a concentration at which no significant effect of hypotonicity on cleavage rate is observable. The controls, moreover, were in the same concentration of sea water as the experimental eggs were.

Two brands of methylene blue were used, LaMotte and National Aniline, the majority of the experiments being performed with the latter. In most of the experiments the eggs had already been in the dye solutions for approximately 10 minutes before insemination, in two experiments for 15 and 43 minutes, respectively. The eggs were immersed in the dye for a period prior to fertilization to insure that penetration into the cell had occurred and that the cells were already at temperature equilibrium. The whole experimental procedure enables the observer to detect relatively small differences in cleavage time.

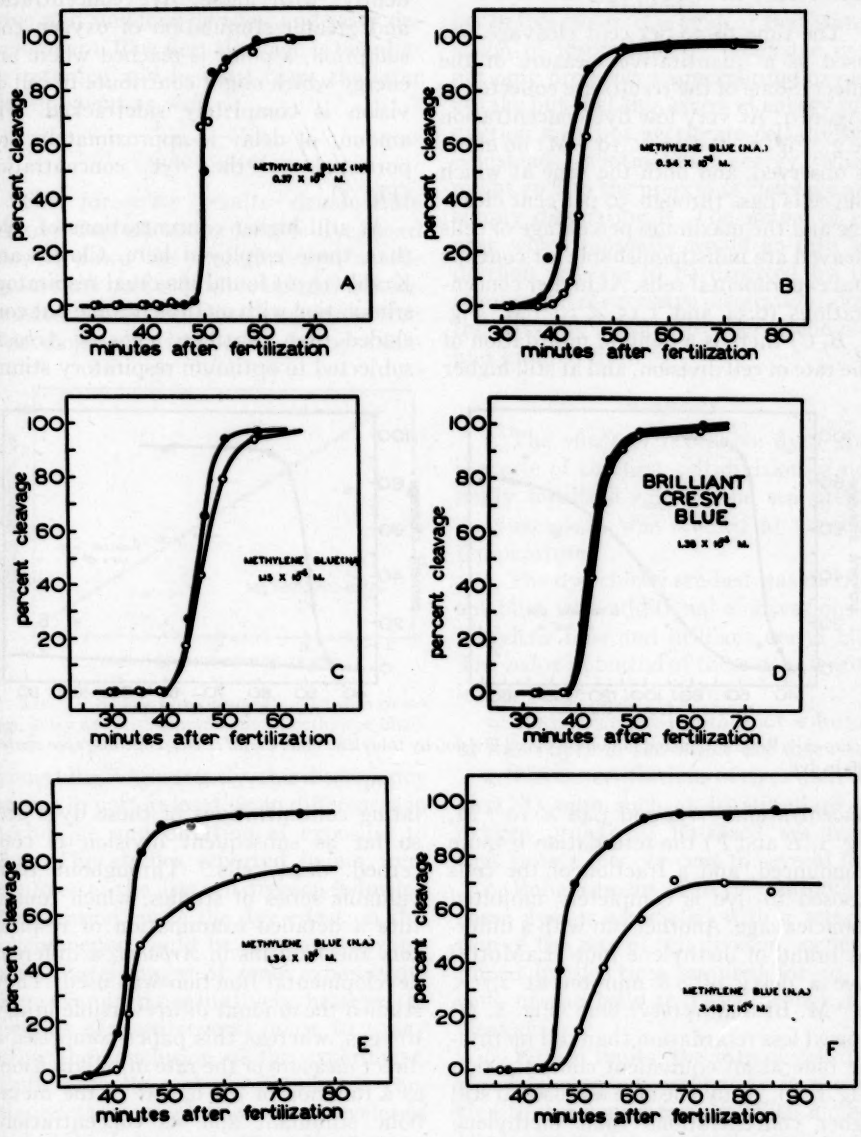

FIG. 1.—Slowing of cell division by methylene blue (National Aniline), in various concentrations (*A*, *B*, *C*, *E*, and *F*) and by brilliant cresyl blue (*D*). *Closed circles*, controls; *open circles*, cells in dye.

RESULTS

The time to 50 per cent cleavage was used as a quantitative measure of the effect. Some of the results are collected in Figure 1. At very low dye concentration (e.g., Fig. 1, *A*, 0.37×10^{-4} M) no effect is observed, and both the time at which the cells pass through 50 per cent cleavage and the maximum percentage of cells cleaved are indistinguishable for controls and experimental cells. At higher concentrations (0.54 and 1.15×10^{-4} M, Fig. 1, *B*, *C*) there is a definite retardation of the rate of cell division, and at still higher concentrations (1.34 and 3.48×10^{-4} M, Fig. 1, *E* and *F*) the retardation is more pronounced, and a fraction of the cells exposed to dye is completely inhibited from cleavage. Another run with a different brand of methylene blue (LaMotte) gave a delay of 8.8 minutes at 2.1×10^{-4} M. Brilliant cresyl blue (Fig. 1, *D*) showed less retardation than did methylene blue at an equivalent concentration (Fig. 1, *E*). Toluylene blue was used at still higher concentrations than methylene blue (Fig. 2). At 4.41×10^{-4} M cleavage was delayed approximately 52 minutes, whereas at 7.13×10^{-4} M, a maximum of 5 per cent of the cells underwent division (mostly abnormal cleavages) during a 110-minute period of observation. Evidently, with higher dye concentration and greater stimulation of oxygen consumption, a point is reached where any energy which might contribute to cell division is completely sidetracked. The amount of delay is approximately proportional to the dye concentration (Fig. 3).

At still higher concentrations of dyes than those employed here, Clowes and Krahl (1936) found maximal respiratory stimulation with methylene blue but concluded that "fertilized eggs of *Arbacia* subjected to optimum respiratory stimulating concentrations of these dyes are, so far as subsequent division is concerned, dead cells." Throughout their valuable series of studies, which constitute a detailed examination of respiratory mechanisms in *Arbacia*, a different developmental function was used. They studied the amount of irreversible injury to eggs, whereas this paper comprises a direct measure of the rate of cell division, as a function of the nature of the metabolic stimulant and its concentration. Cells which are irreversibly blocked are, according to these authors, "so far as subsequent divisions are concerned, dead cells." In the present investigations it is shown that working in the region where one obtains significant increases in the

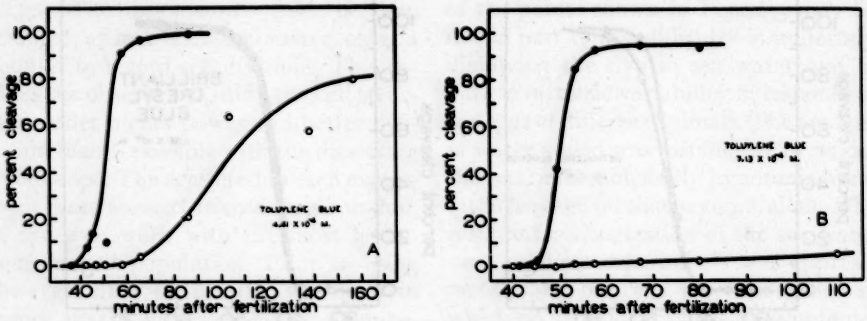

FIG. 2.—Retardation and inhibition of cell division by toluylene blue. *Closed circles*, controls; *open circles*, cells in dye.

rate of oxygen uptake, the cells will survive and undergo division and later development. The first cleavage is definitely retarded and in most cases the later development as well.

DISCUSSION

The foregoing results demonstrate clearly that all the reversible dyes used slowed down the first cleavage, at the same time that oxidative uptake was enhanced. M. M. Brooks (1943) states that "development was accelerated by methylene blue." Apparently, this discrepancy stems, in part at least, from differences in technique and duration of exposure to dye. The studies reported herein were confined to the first cell division following fertilization, since the dye effect on this phenomenon could be accurately evaluated. Later stages of some experiments were examined qualitatively, however, at periods of from several hours to a day after the conclusion of the experiment. Both controls and dyed embryos continued to segment and often developed normally. In general, where there was a pronounced retardation of first cleavage, there was more or less retardation of later developmental stages in the dyed embryos, but this point should be examined more exhaustively.

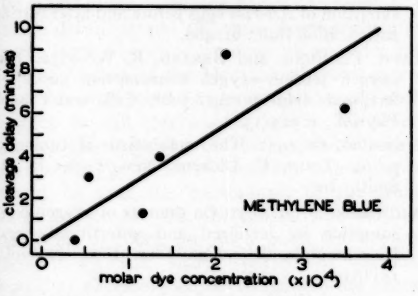

FIG. 3.—Showing proportionality between cleavage delay and dye concentration (methylene blue).

To return to the questions raised earlier in this paper: it is evident that stimulation of respiration by reversible dyes not only produces a supernormal oxygen uptake but that this excess of energy production does not accelerate cell division—instead, sidetracking energy which might go into the process of cleavage and simply dissipating it. The source of energy which normally would go into cell division appears to be diverted by the stimulation of excessive respiration, along the pathway affected by methylene blue and kindred dyes.

SUMMARY

1. The effect of reversible dyes upon the rate of the first cell division of normally fertilized eggs of the sea urchin, *A. punctulata*, was studied at constant temperature.

2. The dye chiefly studied was methylene blue, with additional observations on toluylene blue and brilliant cresyl blue. The redox potential of these dyes is within the range +11–115 mv.

3. Some technical details of solutions of these dyes in sea water are discussed.

4. The concentrations of dyes used (*ca.* 10^{-4} M) were such as to stimulate the oxygen uptake of fertilized sea-urchin eggs from a few per cent to several fold.

5. Enhancement of oxygen uptake by these dyes is associated with a retardation of the rate of cell division, as determined by the time required for 50 per cent of the cells to pass through first cleavage.

6. Within limits, the rate of cell division is slowed approximately in proportion to dye concentration. With increasing concentration, complete inhibition of the division of an increasing percentage of cells is observed.

7. Simply increasing the metabolism of the cell does not lead to acceleration of cell division. The increased energy must

be channeled in a given direction in order to facilitate cell division. As these results show, by the use of suitable agents (dyes), energy normally going into cell division may be diverted, with resultant retardation of cell division.

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SOME EFFECTS OF NARCOSIS ON THE NERVE IMPULSE IN THE SQUID, LOLIGO OPALESCENS

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IN THEIR study of the blocking effects of pressure and of cocaine on the sciatic nerve of the bullfrog and the saphenous nerve of the dog, Gasser and Erlanger (1929) found some remarkable differences in the behavior of fast and slow fibers. Their conclusions may be summarized as follows: (1) The relative susceptibility of the fibers to pressure and to cocaine is, in general, determined by the size of the fiber, although the differentiation is not precise. Pressure produces block first in the large, then in the small, fibers, while cocaine affects the small fibers first and the larger ones later. Recovery takes place in reverse order, i.e., large fibers recover first from cocaine narcosis, small ones later. (2) Block is anteceded by a great retardation in con-

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duction velocity. (3) It is not necessary to make use of the difference in thickness of the myelin sheaths in formulating an explanation. If it is assumed that the cocaine acts by chemical combination with the protoplasm, then, since the surface per unit volume increases directly as the diameter decreases, the smaller the fiber, the greater the accessibility.

In reference to the fact of recovery in reverse order, Gasser and Erlanger say:

> At first sight, such an hypothesis suggests that when the nerve is washed with physiological saline the same factor would also cause the small fibers to be the first to recover. But this is definitely not the case. Recovery begins in the large fibers and proceeds in an order the reverse of that of the blocking, thereby corresponding with what has long been known with respect to the return of function. However, further consideration of the accessibility hypothesis suggests that a possible consequence would be that the protoplasm of the small fibers combines with the poison not only to the point of block but to a point far beyond this. . . . Thus a longer period of washing would be necessary either to remove the excess drug or to allow more time for reorganization.

It seemed possible to gain new light on this problem by studying the narcosis of nerves of invertebrates in which the sheath is very thin as compared with the myelin sheath of the vertebrates. The possibilities of the cephalopod nerve have been explored by Pumphrey and Young (1938). They list the fiber sizes of nerves in the squid, *Loligo pealii*, and their results show that conduction velocity varies as the square root of the diameter, a relation which differs somewhat from the usual rule in vertebrates. None of the fibers in the squid nerve has a fatty sheath stainable with osmium tetroxide, but under polarized light they were all seen to be surrounded by a very thin, myelin-like sheath (Bear, Schmitt, and Young, 1937). This difference in the sheath form, between cephalopods and vertebrates, seems sufficient to warrant a re-examination of the problem of the relation of fiber size to conduction rate and amplitude under narcosis.

MATERIALS AND METHODS

All experiments were carried out on the fin nerve of *L. opalescens*. The following drugs were used in sea-water solution: procaine $\frac{1}{3}$ per cent and 1 per cent, urethane 2 per cent, chloretone half-saturated solution, and ether 5 per cent. The solution of narcotic was applied to the nerve midway between the stimulating and the recording electrodes. The nerve was maintained in a moist chamber on a series of silver electrodes set up in the usual fashion. To produce monophasic impulses, one end of the nerve was killed by crushing and was arranged so that the killed area was over the distal pickup electrode. The conduction distance was constant at 1.6 cm. Stimuli about 0.1 msec. in duration were delivered at a rate of approximately one per second from a thyratron stimulator. Action potentials were led through a conventional two-stage preamplifier to a DuMont No. 208 oscilloscope, the sweep of which was triggered from the stimulating circuit.

RESULTS

The oscillographic records of the action potential of normal fin nerves show a large initial wave (left) followed by a lower one (right), with an intervening gap indicating that between the fast fibers and the slower ones there are very few of intermediate rate (Figs. 1–4). In view of the studies of Pumphrey and Young (1938) on the nerves of a closely related form (*L. pealii*), it seems reasonably certain that the first wave (left) of the action potential of the fin nerve represents impulses from the larger, faster fibers of the nerve, while the second wave

results from impulses from the smaller, slower fibers. Also, analogy with the situation in Crustacea suggests that the fast fibers may be predominantly motor in function, the slow fibers mainly sensory.

The first sign of narcosis in the fast fibers is, as a rule, depression of potential with no change in rate of conduction (Figs. 1, 2). However, the velocity does diminish in some cases with the reduction in potential (Fig. 4). A drop in potential in the slow fibers is, as a rule, associated with a slowing in rate (Figs. 1, 3) but may occur without it (Fig. 4). Similar variability has been reported for the fin nerve of *L. pealii* by Bullock, Nachmansohn, and Rothenberg (1946). Evidence that reduction in potential represents a true effect of the narcotizing agent, i.e., blocking of the impulse in certain fibers and not a simple shunting effect of the solution applied is shown by the recovery of the nerve after the poisoned area was washed with sea water (Figs. 2 and 4).

Figure 1 (procaine narcosis) is typical of the results obtained. Reduction in height of the potential (left) of the fast fibers occurs without any change in rate, but for the slow fibers great reduction of potential (right) is accompanied by a

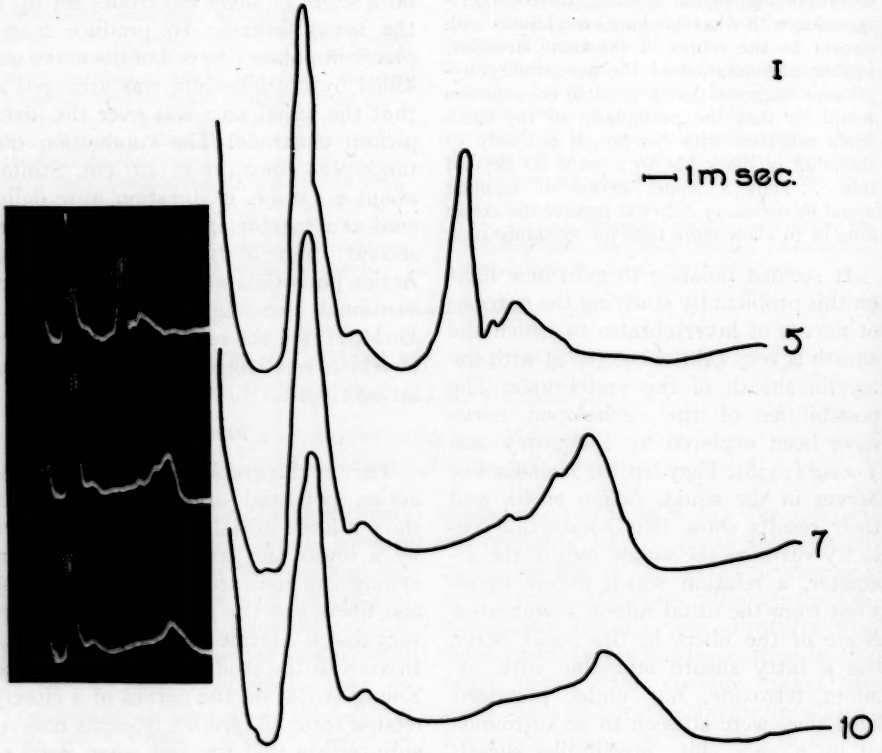

FIG. 1.—*5*, normal. *7*, procaine ¼ per cent ½ minute. *10*, procaine ¼ per cent 2 minutes. Fast wave at left, slow wave at right. Procaine narcosis causes a reduction in the height of potential of the fast fibers without a change in rate. In the slow fibers there is great reduction of potential with great decrease in rate. Retardation does not precede reduction of potential but accompanies it.

corresponding decrease in rate. Thus, while the rate of the fast fibers is normally 16 m.p.s. and remains so under narcotization, the slow fibers show an initial rate of 2.5 m.p.s.; a rate of 1.65 m.p.s. for the first stage in narcotization; and for the last stage a rate of 1.3 m.p.s. There is no indication that reduction in potential is preceded by a slowing of the rate; the two effects proceed *pari passu* in the slow fibers. In two respects, then, the nerve fibers of the squid differ from those of the frog and the dog in their reactions to cocainization; (1) the fast fibers of the squid do not show any slowing in rate with narcotization, whereas the fibers of the vertebrate nerves do; (2) the slow fibers are the most strongly affected in both animal types, but in the squid retardation of the impulse does not precede the reduction of potential but accompanies it.

In Figure 2 (ether narcosis) it is apparent that significant reduction in the potential of the fast fibers is not accompanied by any slowing in rate. This figure shows almost complete recovery in the fast fibers before there is any evidence of recovery in the slow ones. This confirms the finding of Gasser and Erlanger that fast fibers recover first.

Figure 3 (urethane narcosis) shows reduction in height of potential and rate of conduction in the slow fibers with no significant change in either respect in the fast fibers. This confirms the conclusion of Gasser and Erlanger (1929) that slow fibers are first affected.

Figure 4 shows an effect sometimes obtained by narcotization with procaine

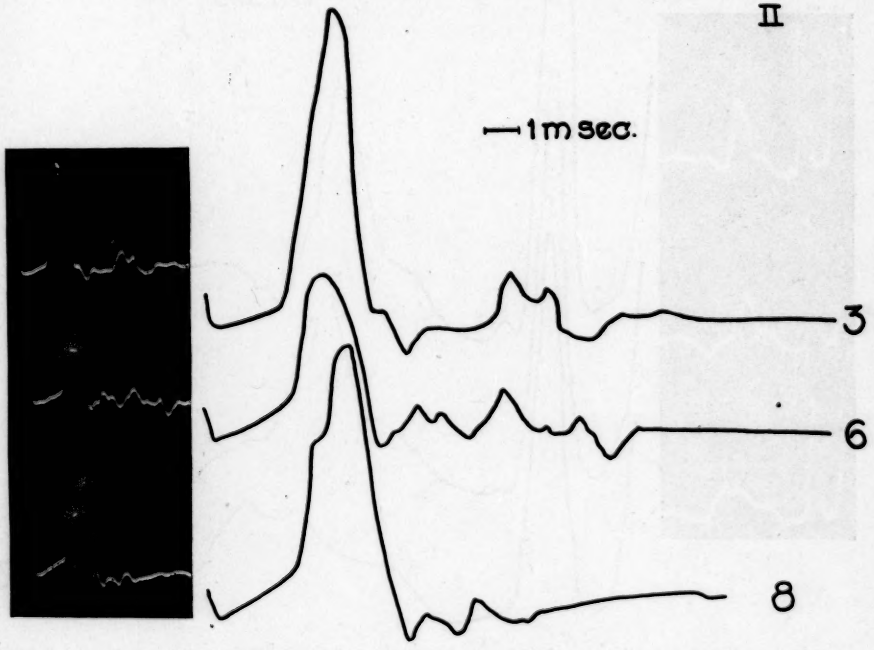

FIG. 2.—3, normal. 6, 5 per cent ether 1 minute. 8, recovery in sea water 3 minutes. Ether narcosis leads to reduction of potential in the fast fibers without slowing of the rate. Recovery takes place in the fast fibers before there is evidence of recovery in the slow ones.

after recovery from treatment with ether. Recovery from procaine takes place more rapidly in the fast than in the slow fibers. In respect to changes in conduction rate and potential of the fast fibers, the results parallel those of Gasser and Erlanger on vertebrate nerve.

DISCUSSION

In explaining the effects of narcotics on mixed nerve fibers, other possibilities exist besides the "accessibility hypothesis" of Gasser and Erlanger (1929). It is, for example, possible that, since the sheaths of the large fibers in vertebrates are thicker than those of the small fibers, narcosis may depend upon the penetration of this sheath before the narcotic can affect the active, i.e., conducting, surface. This would account for the order in which narcosis takes place, namely, small fibers first, large ones later. Since in recovery the large fibers are first, it may be supposed that normal function is resumed here as soon as the molecules of poison have diffused from the surface layer into the sheath. The deeper penetration of the small fibers makes necessary, in recovery, a longer time for the

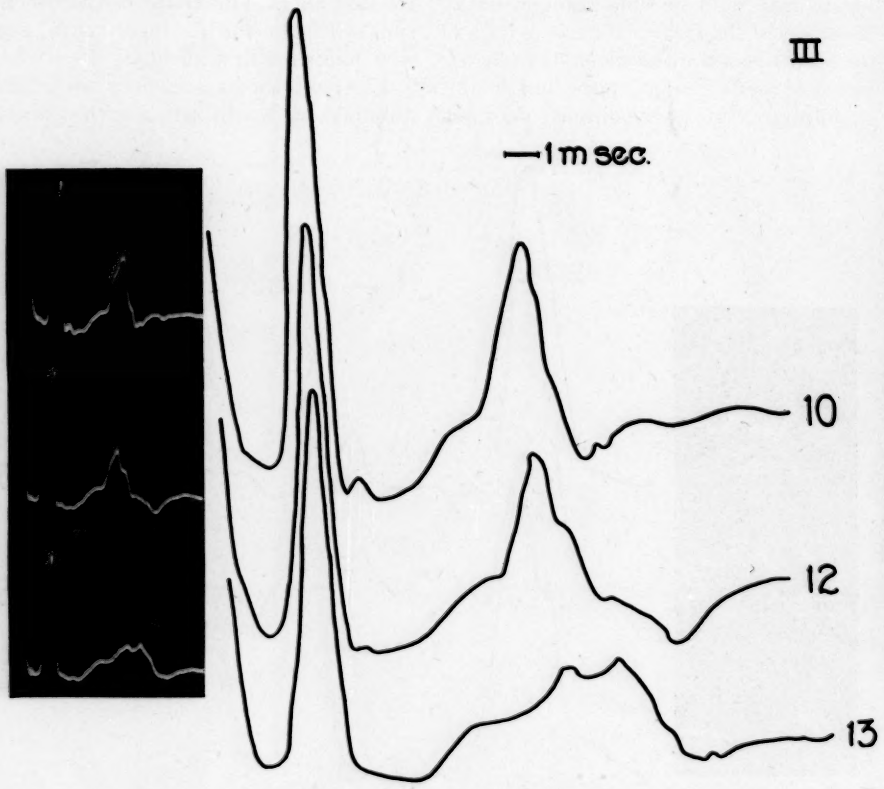

FIG. 3.—*10*, normal. *12*, urethane 2 per cent 1 minute. *13*, same for 5 minutes. Urethane narcosis shows reduction of potential and rate of conduction in the slow fibers with very slight reduction of potential and no change in rate in the fast fibers.

poison finally to leave the surface layer of the relatively unsheathed fiber.

There appear to be at least two ways in which the protoplasm may participate in the reaction: (1) electrically, by alterations mainly in resistance, according to the scheme of Curtis and Cole (1940); (2) indirectly, as a result of the function of the cytoplasm serving as a reservoir of material for the restoration of the surface. Any immediate dependence of the surface on the core is rendered questionable by the experiments of Hodgkin and Huxley (1939) and Curtis and Cole (1940), in which glass electrodes were inserted into the core of the giant fiber of the squid; conduction was not affected unless the underside of the outer membrane was injured.

On the specific problem of the action of nerve toxins, Welsh and Gordon (1947) have suggested, as a result of their work with various insecticides, that the poison exerts its effect on nerve by means of a reaction with the surface (lipid) layer of the fiber. They did not attempt to determine the relation of fiber size to speed of action of the poison. Toman, Woodbury, and Woodbury (1947) propose an increase in threshold of excitability as the underlying factor in the block of the action potential by procaine, eserine, and di-isopropyl fluorophosphate.

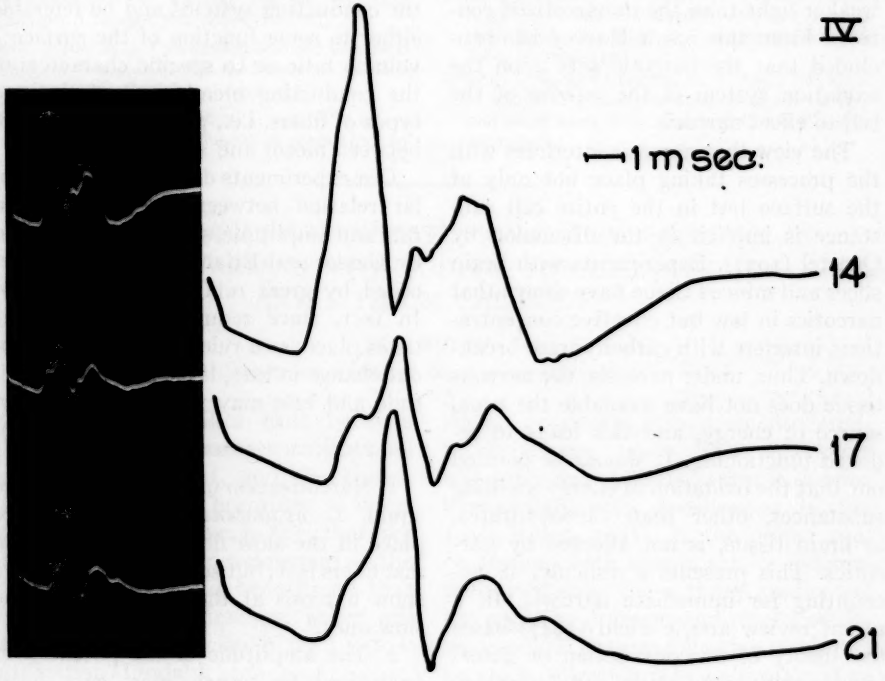

FIG. 4.—*14*, normal after recovery from ether. *17*, procaine 1 per cent 2½ minutes. *21*, recovery in sea water, 4 minutes. Narcotization with procaine after ether is equally effective on fast and slow fibers, but recovery of potential and conduction rate are shown by the fast fibers, while the slow fibers do not give evidence of recovery in either respect.

The problem is a part of the general one of cellular narcosis: whether the poison acts solely on the cell membrane or on the cell body as well. Harvey's (1940) experiment on the narcotization of luminescence in *Noctiluca* bears on the point. In this form, luminescence occurs only in the presence of oxygen. Under ether narcosis, luminescence fails. This could be caused by the ether's preventing the penetration of oxygen through the membrane or by narcosis of the cellular protoplasm itself. Harvey broke down the etherized cells with sand so that the membranes were destroyed and the luminescence system had free access to oxygen. When thus fragmented, the etherized preparation always gives much weaker light than the unnarcotized controls. From this result Harvey has concluded that the narcotic acts upon the oxidation system of the *interior* of the cell to effect narcosis.

The view that narcosis interferes with the processes taking place not only at the surface but in the entire cell substance is implicit in the discussion by Quastel (1937). Experiments with brain slices and minced tissue have shown that narcotics in low but effective concentrations interfere with carbohydrate breakdown. Thus, under narcosis, the nervous tissue does not have available the usual source of energy, and this leads to reduced functioning. It should be pointed out that the oxidation of energy-yielding substances, other than carbohydrates, in brain tissue, is not affected by narcotics. This presents a difficulty in accounting for immediate narcosis. In a recent review article Field (1947) bases the theory of narcotic action on interference with respiration and fermentation. Such a theory accords with the view that the entire nerve substance is concerned in narcosis. Caution should, however, be used in applying information derived from brei, cells, and brain-slices to explain phenomena in the nonnucleated axis cylinder.

The striking difference in the behavior of the two groups of nerve fibers in the squid calls for an explanation either on the basis of the specific nature of the membranes or in the constitution of the axis cylinder itself. Since, in the squid nerve, myelinization of fibers of all types is light, it can hardly be supposed that this factor alone is responsible for the greater susceptibility of the slow, as compared to the fast, fibers. This greater responsiveness to narcotics on the part of the slow fibers and relative lack of reactivity of the fast fibers must then be the result of differences in the nature of the conducting systems and be referable either to some function of the surface / volume ratio or to specific characters of the conducting membranes of the two types of fibers, i.e., possibly a difference between motor and sensory fibers.

Our experiments did not reveal a regular relation between the reduction in rate and amplitude, such as that reported by Gasser and Erlanger, viz., block preceded by great retardation in velocity. In fact, since reduction of amplitude takes place, as a rule, in fast fibers without change in rate, it is clear that amplitude and rate may vary independently.

SUMMARY

1. Narcotization of the fin nerve of the squid, *L. opalescens*, in general, takes place in the slow fibers first and in the fast fibers last; but some of the fast fibers show narcosis at the same time as the slow ones.

2. The amplitude of the potential in narcotized fin nerves may vary independently of the rate of conduction.

3. Retardation of rate does not precede reduction of potential.

4. Since, in the squid, myelinization of

all the fibers is extremely light, that factor cannot be assumed to account for differences in the susceptibility of fast (large) and slow (small) fibers. The variation must then be due to differences in the nature of the conducting systems, which, in turn, may be referred either to some function of the surface/volume ratio or to specific differences in the constitutions of the two types of fiber.

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THE CONTROL OF RECONSTITUTIONAL DEVELOPMENT IN PLANARIANS WITH SODIUM THIOCYANATE AND LITHIUM CHLORIDE[1]

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MANY similarities exist between embryonic and reconstitutional development, and the knowledge of one type has been used to interpret various phenomena in the other (see Child, 1941). In the development of the echinoderm egg it has been found that NaCNS in calcium-free sea water will produce ectodermization if used before fertilization (Lindahl, 1933, 1936; Rulon, 1941). On the other hand, LiCl, under similar conditions, produces entodermization (Rulon, 1946). Both substances will produce entodermization if applied immediately after fertilization (Child, 1936; Rulon, 1941). In the proper concentrations both agents act by differentially inhibiting the activity gradient of the egg. The different end-results on developmental pattern following treatment before fertilization may be explained on the basis of more rapid recovery, but with loss or decrease of correlative factors, from the effects of NaCNS. Since these agents are so effective in modifying

[1] The experimental portion of this work was done at the University of Chicago during the summer of 1940. For the use of laboratory space and facilities the writer is most grateful.

echinoderm development, it seems important to determine their actions on reconstitutional processes.

MATERIALS AND METHODS

The planarians (*Dugesia dorotocephala*) were collected in large numbers from a spring near Cary, Illinois, and maintained in the laboratory under standard conditions (see Rulon, 1936). Animals 14–16 mm. in length and starved for at least 1 week were used, and for these particular studies only short pieces from a region low in physiological activity were considered. The pieces were approximately 1 mm. in length (one-sixteenth of total body length) from the region immediately posterior to the mouth (Fig. 1, *I and J*).

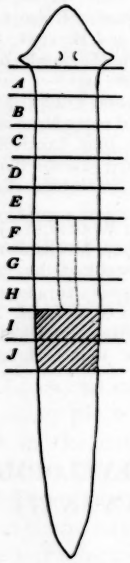

FIG. 1.—Sections approximately one-sixteenth the length of the intact animal are designated as *A*, *B*, *C*, etc. Only sections *I* and *J* (*shaded*) from the region immediately posterior to the mouth are used in these experiments.

In all experiments, lots of fifty pieces were used. These lots were obtained by sectioning sixty-five animals and selecting fifty pieces of most nearly the same size from each region. The pieces were then placed in 150 cc. of the test solutions or well water in covered finger bowls for varying intervals of time after section. All tests were controlled by the reconstitution of similar pieces in well water.

The reconstituted animals were examined at approximately 10 days after section, and records were made of the degree and type of development. Classification of these forms was made in the following manner:

A. Head types[2] and tail regeneration.
   - I*a*. Normal or enlarged triangular heads, with auricles at lateral margins and two distinct eyespots. Tails inhibited or absent.
   - I*b*. Normal or enlarged triangular heads, with auricles at lateral margins and two distinct eyespots. Normal tails.
   - II. Teratophthalmic. Triangular heads, with auricles in normal position but with eyes showing all degrees of approximation to the median plane, from two distinct eyes with pigment connected up to complete cyclopia. Normal tails.
   - III. Teratomorphic. Head more or less rounded in outline, with auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle and with a single, or apparently single, median eye. Normal tails.
   - IV. Anophthalmic. Head rudimentary, without eyes and with or without a single median auricle. Normal tails.
   - V. Acephalic. Head completely absent. Normal tails.

B. Bipolar. Head regeneration at both the anterior and the posterior cut surfaces.

C. Apolar. Heads and tails completely absent.

D. Dead. Pieces dying during course of experiment.

E. Head-frequency index. An approximate "average" for degree of head regeneration for lots of pieces from any particular level.

[2] This series of head forms in *Dugesia* has been repeatedly described and illustrated by various authors (see Child, 1941, p. 178).

This is arrived at by giving to each head type a number which approximates its degree of inhibition (normal = 100; teratophthalmic = 80; teratomorphic = 60; anophthalmic = 40; acephalic = 20). The actual number of each head type for any particular level is then multiplied by the number corresponding to its degree of inhibition, and the sum of all is divided by the total number of animals reconstituting from that level.

EXPERIMENTAL DATA

1. *The effect of lithium treatment before and after section.*—Whole animals were cent were headless. The same general results were obtained for *J*-levels.

Lithium before section caused not only an increased head frequency but also an increased scale of organization. This was shown particularly by the increase in forms with normal heads but inhibited tails (Table 1, I*a*) at both *I*- and *J*-levels. It was also interesting to note that this treatment gave an increase in percentage of bipolar forms.

When short posterior pieces from untreated animals were placed in a well-

TABLE 1

THE EFFECTS OF LITHIUM CHLORIDE ON HEAD FREQUENCY AND SCALE OF ORGANIZATION IN POSTERIOR ONE-SIXTEENTH PIECES OF *D. dorotocephala*

(Values in Percentages)

Experiments	Level	Head Types*						Bipolar	Apolar	Dead	Head-Frequency Index
		Ia	Ib	II	III	IV	V				
Well water (control)....	I	6	8	1	1	11	43	3	12	15	40
	J	2	8	8	4	11	49	0	11	7	38
M/3,000 LiCl, 3–5 days before section.....	I	14	20	3	1	9	9	10	9	25	72
	J	11	30	1	1	15	17	4	6	15	67
Well water (control)....	I	0	18	0	0	6	64	2	6	4	38
	J	0	20	8	0	8	58	0	4	2	43
M/3,000 LiCl, 3 days after section.....	I	0	4	4	2	28	60	0	0	2	32
	J	0	16	0	4	14	62	0	0	4	38

* Ia, normal heads, inhibited tails; Ib, normal heads, normal tails; II, teratophthalmic, normal tails; III, teratomorphic heads, normal tails; IV, anophthalmic, normal tails; V, Acephalic, normal tails.

placed in a well-water solution of LiCl (M/3,000) for 3–5 days, after which they were sectioned and allowed to reconstitute in well water. The results are shown in Table 1. Short posterior (*I*, *J*) pieces from animals treated with lithium before section gave a definite increase in head frequency (compare head-frequency indices of test and control). Thirty-four per cent of the test animals from the *I*-level regenerated normal heads, while only 9 per cent were headless (acephalic). In the control from this level, only 14 per cent had normal heads, while 43 per

water solution of M/3,000 LiCl for the first 3 days after section and then returned to normal well water for the remainder of the reconstitutional period, the pieces gave little evidence of either an increased head frequency or an increased scale of organization. In fact, both *I*- and *J*-levels gave some evidence of a decrease in head frequency (Table 1, head-frequency indices).

2. *The effect of thiocyanate treatment before and after section.*—Intact animals were placed in a well-water solution of M/2,500–M/3,000 NaCNS for 3–5 days,

after which they were sectioned and allowed to reconstitute in well water. Examination of the small reconstituted forms showed a slight decrease in head frequency at both *I*- and *J*-levels (Table 2).

When untreated animals were sectioned and the pieces placed directly in M/2,500 NaCNS for the first 3 days following section, a considerable increase in head frequency was noted at both levels studied (Table 2). There was also an increase in bipolar forms but little or no increase in animals with normal heads and inhibited tails.

3. *The effect of treatment with lithium before and thiocyanate after section.*—The most outstanding effects of all came by exposing the intact animals to M/3,000 LiCl for 3–5 days, after which they were sectioned and the short *I*- and *J*-pieces permitted to reconstitute for the first 2–3 days after section in M/2,500–M/3,000 NaCNS. After the exposure to thiocyanate, the pieces were placed in well water for the remainder of the reconstitutional period. This treatment was extremely severe, and the death rate was high, as shown in Table 3 (51 per cent for *I*-pieces; 34 per cent for *J*-pieces). Most of the living animals had normal heads. There was a decrease in number of apolar forms and a considerable increase in bipolars at both levels. Approximately one-third of the forms developing from the *I*-level were bipolar. The scale of organization was vastly increased. This was shown particularly by an increase in forms with inhibited tails but normal heads. In general, the picture of development for this level resembles that of a level far anterior (level *A* or *B*, Fig. 1).

TABLE 2

THE EFFECTS OF SODIUM THIOCYANATE ON HEAD FREQUENCY AND SCALE OF ORGANIZATION IN POSTERIOR ONE-SIXTEENTH PIECES OF *D. dorotocephala*

(Values in Percentages)

Experiments	Level	Head Types*						Bipolar	Apolar	Dead	Head-Frequency Index
		Ia	Ib	II	III	IV	V				
Well water (control)	I	0	7	0	2	9	67	1	14	0	29
	J	0	2	1	1	18	69	0	6	3	26
M/2,500–M/3,000 NaCNS, 3–5 days before section	I	1	0	0	2	7	71	1	9	9	24
	J	0	0	0	1	9	82	0	3	5	22
Well water (control)	I	0	18	0	0	6	64	2	6	4	38
	J	0	20	8	0	8	58	0	4	2	43
M/2,500 NaCNS, 3 days after section	I	2	38	2	2	6	32	14	0	2	68
	J	0	46	4	0	10	24	4	0	12	70

* For explanation of head types see note to Table I.

DISCUSSION

All levels of *Dugesia* will regenerate a head if only a single section is made. It has been found, however, that the rate of head formation is more rapid at anterior than at posterior levels of the first zoöid (Watanabe, 1935). Under normal conditions short transverse pieces from posterior levels usually develop inhibited heads or no heads at all, and this has been shown to be due to certain inhibitory factors which arise at the site of posterior section in a piece and extend for-

ward, possibly by way of the ventral nerve cords (Buchanan, 1922; Child and Watanabe, 1935). A head develops on a piece only if the head-forming region of that piece can escape the inhibitory influence of the posterior cut surface.

Short anterior pieces commonly regenerate 100 per cent normal or near-normal heads. This seems to be because the pieces are from regions high in the physiological gradient, where the head-forming cells are more active and the does not reappear after return to water, the head-forming cells are free from its action. Some increase in head frequency may also result from differential conditioning with continuous exposure to still lower concentrations of the depressing agent.

In the experiments described in the present paper it has been shown that when lithium is used only before section (Table 1) there is an increase in head frequency, scale of organization, and bi-

TABLE 3

COMBINED EFFECTS OF LITHIUM CHLORIDE AND SODIUM THIOCYANATE ON HEAD FREQUENCY AND SCALE OF ORGANIZATION IN POSTERIOR ONE-SIXTEENTH PIECES OF *D. dorotocephala*

(Values in Percentages)

Experiments	Level	Head Types*						Bipolar	Apolar	Dead	Head-Frequency Index
		Ia	Ib	II	III	IV	V				
Well water (control)....	I	6	8	1	1	11	43	3	12	15	40
	J	2	8	8	4	11	49	0	11	7	38
M/3,000 LiCl, 3–5 days before section, M/2,500–M/3,000 NaCNS 2–3 days after section.....	I	17	5	4	1	1	0	16	5	51	88
	J	18	20	9	0	2	3	12	2	34	90

* For explanation of head types see note to Table 1.

posterior head-inhibiting factors are less effective. Posterior pieces usually develop into forms with no heads or grossly inhibited heads, presumably because at posterior levels the head-forming cells are less active and the head-inhibiting factors are more effective. It has been shown by a number of investigators[3] that the head frequency of short posterior pieces may be greatly increased by temporary exposure, following section, to slightly depressing concentrations of various agents, after which the pieces are returned to water and allowed to recover. Since the inhibiting effect of posterior section lasts only a few hours and

[3] See Child (1941) for the most recent review on the control of correlative factors in planarian reconstitution.

polarity in short posterior pieces. This suggests that lithium, on penetrating the intact animal, produces a block which prevents, at least in part, the influence of one cut surface upon the other. Under these conditions the head-forming region of a piece, no longer under the direct inhibitory action of lithium and freed to a large extent from the effects of posterior section, tends to recover, often with an increased scale of organization. The increase in bipolar forms may be explained in part by a decrease in correlating factors in the piece brought on by lithium and also by the fact that the piece is from a region near the fission plane, where activation of posterior cells to form heads is more likely to occur.

When pieces from untreated animals

are exposed to lithium for the first 3 days after section, there may be a slight decrease in head frequency (Table 1). It would at first appear that this decrease (if significant at all) may be due to direct action of the agent on the head-forming cells. However, more recent work (Rulon, unpublished) in which posterior halves, with only an anterior cut surface, were exposed continuously to M/1,500 LiCl does not support this view. Approximately 90 per cent of the pieces exposed to this high concentration cytolyzed, yet the small number that did tolerate it, although somewhat swollen in appearance, regenerated, while still in the solution, normal heads and approximately the same amount of new tissue as the controls. The factors which may cause a decrease in head frequency in short posterior pieces after 3 days' exposure to LiCl are obscure. Under the conditions of these experiments it appears that the posterior inhibitory factors are even more effective than in the controls.

The data show that treatment with NaCNS before section produces effects entirely different from those caused by lithium under similar conditions (Table 2). Instead of blocking the posterior influences, it appears either to increase them or to decrease the activity of head-forming cells. It may well be that treatment of the intact animal with this agent causes a general flattening of the physiological gradient system. On return to well water and sectioning, differential recovery processes may lead to an even steeper gradient than before and with this a decrease in head-forming activity at posterior levels.

The exposure of short posterior pieces from untreated animals to NaCNS for 3 days after section (Table 2) increases head frequency. Under these conditions this agent appears to act chiefly as a block to posterior inhibiting factors while the head-forming regions recover. Support is given to this view from other experiments (Rulon, unpublished), in which posterior halves, with only anterior cut surfaces, were exposed for 8 days to M/1,500 M NaCNS. At the end of this time 90 per cent of the pieces were still alive but were curled up in the bottom of the dish and gave only slight response to stimulation. Only a slight amount of new head-forming tissue had developed, and there was no evidence of either eyespots or auricles, although the controls had all developed normal heads. When these thiocyanate-treated forms were returned to water, all developed normal heads within a few days.

In the final experiments, when intact animals were exposed to lithium before section and the pieces to thiocyanate after section, it was noted that the effects of these agents were additive (Table 3). This combination treatment was apparently so effective in blocking the effects of posterior cut surface that the pieces tended to regenerate a head of the size and capacity for dominance that the level would have given if no posterior section had been made. Under such conditions the scale of organization was too large for the piece, and many tailless forms appeared. Loss of correlating conditions and proximity of the fission plane probably account for the high percentage of bipolar forms arising from this drastic treatment.

SUMMARY

1. Lithium chloride and sodium thiocyanate were used before and after section in modifying reconstitution in short transverse pieces (one-sixteenth the length of 14–16-mm. animals) of *D. dorotocephala* from the region immediately posterior to the mouth.

2. Treatment with M/3,000 LiCl for 3–5 days before section leads to an increased head frequency and an increased

scale of organization in short posterior pieces. Treatment after section gives a slightly decreased head frequency.

3. Treatment with M/2,500–M/3,000 NaCNS for 3–5 days before section results in a slightly decreased head frequency but, after section treatment of the pieces for 3 days, causes a markedly increased head frequency.

4. A double treatment with lithium before, and thiocyanate after, section gives a very high head frequency, a great increase in scale of organization, and a large percentage of bipolar forms.

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CHANGES IN THE DISTRIBUTION OF NITROGEN IN THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN) DURING METAMORPHOSIS[1]

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IN THE classical concept, metamorphosis in holometabolous insects is accompanied by histolysis of mature larval structures, followed by construction of the tissues and organs of the adult. Beginning with Weismann (1864), this fact has been demonstrated by dissection and by histological techniques; in addition, repeated attempts have been made, using various species of insects, to determine by analytical methods the exact nature of the chemical changes occurring during the course of the morphological upheaval accompanying metamorphosis. Since the insect pupa is surrounded by a relatively impervious skin, it is evident that only matter in the gaseous state can enter or leave the body; therefore, the adult structures must be constructed, at least in part, of chemical substances made available by the destruction of larval organs. Although

[1] Accepted by the Faculty of the Graduate School of Arts and Science of New York University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The author wishes to express his deep appreciation for the interest, assistance, and helpful criticism of Dr. Daniel Ludwig, under whose direction the work was conducted.

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various investigators have followed the changes in many different compounds during metamorphosis, the fluctuations in the nitrogenous substances would seem to be most directly indicative of the destruction and construction of tissue elements. This is because the tissue framework is largely composed of protein, the stores of fat and carbohydrate being used chiefly as energy sources. Needham (1929) points out that when histolysis of larval structures occurs, their constituent proteins are presumably broken down, resulting in a raised proportion of proteoses, peptones, and even amino acids at the time when histolysis is most intense. As imaginal organs are constructed, this trend should be reversed, and the relative concentration of complete protein should increase. In addition, if some nitrogenous compounds are destroyed during metamorphosis, the fact that nothing may leave the body during the pupal stage should result in an increase in the concentration of the end-products of combustion and in the proportion of nonprotein nitrogen from this source. Needham's review article (1929) gives an excellent summary of the results of analytical work and shows that the postulated conditions do obtain in most cases investigated. For example, Kellner, Sako, and Sawano (1884) found that in the silkworm 100 gm. of dry larval tissue contained 8.11 gm. of protein and peptone nitrogen, 0.29 gm. of chitin nitrogen, and 1.35 gm. of nitrogen in compounds not precipitable by phosphotungstic acid (amino acid nitrogen and excretory nitrogen). In the pupa the same amount of dry tissue contained 5.68 gm. of protein and peptone nitrogen, 0.23 gm. of chitin nitrogen, and 3.25 gm. of nonprecipitable nitrogen. One hundred gm. of dry imaginal tissue contained 8.18 gm. of protein and peptone nitrogen, 0.44 gm. of chitin nitrogen, and 0.87 gm. of amino acid and excretory nitrogen.

Most of the earlier work was concerned with the situation in Lepidoptera, with the exception of Evans' analyses on *Lucilia* (1923) and on *Tenebrio* (1934). In addition, most investigators confined their analyses to larvae, pupae, and adults, without making a complete analytical series during the pupal stage to determine exactly when the changes first occur and when they are reversed. Therefore, it was decided to investigate the changes in nitrogenous components in the Japanese beetle, *Popillia japonica* Newman, and to analyze insects as mature larvae, as prepupae, at stated frequent intervals during the pupal stage, and, finally, as adults. Such a series of analyses is necessary to establish for this species the quantitative data covering the entire period of metamorphosis which were lacking in much of the earlier work on Lepidoptera and which constitute information essential to further investigations into the physiology of metamorphosis in this insect. These data should be of value also in comparative studies of the developmental physiology of insects in general. The Japanese beetle was selected for this work because little appears to have been done with coleopterous insects in this respect. In addition, it is relatively easy to collect and to maintain in the laboratory, and considerable information is available concerning other aspects of its metamorphosis, with which results of the present investigations may be integrated.

MATERIAL AND METHODS

Larvae of the Japanese beetle were collected, brought into the laboratory, and reared in moist soil in individual 1-ounce metal salve-boxes at a temperature of 25° C. They were examined daily and kept plentifully supplied with water

and wheat grains. When an individual reached the stage desired for analysis, it was removed from its box, cleaned of adherent soil, placed in a tared glass vial, and weighed on a Chainomatic balance sensitive to 0.1 mg. It was then placed in a vacuum desiccator over Anhydrone and completely dehydrated under vacuum until it reached a constant dry weight. Dried insects were then stored in a second vacuum desiccator over Anhydrone, under vacuum, until they were analyzed. Vacuum desiccation was used, in order to avoid any possible chemical changes which might be brought about by oven-drying at elevated temperatures.

Insects were thus killed and desiccated as mature larvae, early prepupae, late prepupae, early pupae (day of pupation), late pupae (eighth to tenth day), and adults. In addition, others were taken on successive days of pupal life from the second to the seventh, inclusive. As the length of the prepupal period varies even at a constant temperature, no prepupae between the early and late stages were analyzed. However, at 25° C. the pupal period lasts 10 days, and different pupae killed on the same day of pupal life may be assumed to be physiologically equivalent.

The insects were divided into three series for analysis. One series, consisting of ten larvae, ten early and ten late prepupae, ten early and ten late pupae, ten male and ten female adults, and five pupae from each day of pupal life from the second to the seventh, inclusive, was analyzed for total nitrogen by the Kjeldahl method. A similar series was subjected to fractional analysis for various nitrogenous components. The third series, consisting of six larvae, six early and six late prepupae, six early and six late pupae, three male and three female adults, and three pupae from each of the intermediate pupal days, was analyzed for uric acid. All analyses except those for uric acid were made upon individual insects; in the uric acid series three insects were analyzed together.

ANALYTICAL PROCEDURE

1. *Total nitrogen analysis.*—Each insect, for which the original and the dry weight had been determined, was placed in a 100-ml. Kjeldahl flask and digested with sulphuric acid. The digest was alkalized and distilled, the ammonia evolved being received in 4 per cent boric acid solution containing a standard amount of bromcresol green indicator. This was then titrated with 0.01 N HCl to match a color standard, and the nitrogen originally contained in the insect was arrived at by multiplying by 0.1401 the number of milliliters of standard acid required for the titration (see Niederl and Niederl, 1938, pp. 51–59; Wagner, 1940).

2. *Fractional analysis.*—Each insect was pulverized in the bottom of a 15-ml. centrifuge tube and shaken twice with distilled water to remove all water-soluble compounds. After being shaken, the suspension was separated by centrifugation, and the supernatant solution was filtered through a sintered glass filter with the aid of a vacuum pump. The insoluble residue from centrifugation and filtration was collected and its nitrogen content determined as above. This fraction was termed "Fraction A"; its nitrogen is that of the water-insoluble compounds, complex proteins, and chitin.

The water-soluble nitrogenous substances were contained in the filtrate, known as "Fraction B"; these were then separated further, so that Fraction B does not appear in the results. The solution was treated with tungstic acid to precipitate protein intermediate prod-

ucts (Hiller and VanSlyke, 1922); the precipitate was thrown down by centrifugation, and the nitrogen content of the supernatant fluid was determined as above. This was termed "Fraction C"; it contains the nitrogen of the water-soluble substances in the insect's body not precipitable by tungstic acid, i.e.,

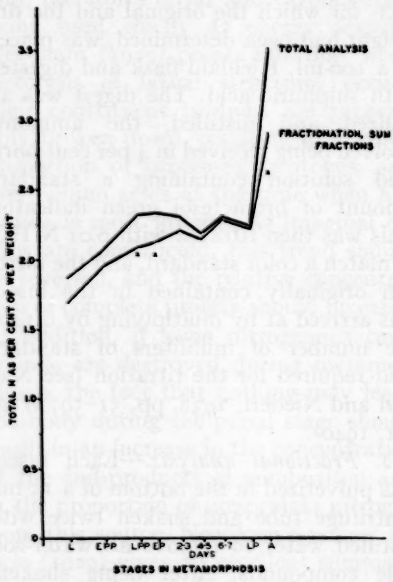

FIG. 1.—Comparison of mean values for total nitrogen, determined by total analysis and by fractionation. Points marked *x* show significant differences. Abbreviations: *L*, larvae; *EPP*, early prepupae; *LPP*, late prepupae; *EP*, early pupae; *LP*, late pupae; *A*, adults.

amino acids, ammonia, urates, and urea, if present.

The tungstic acid precipitate was also analyzed for nitrogen as "Fraction D." This nitrogen consists of that contained in the soluble proteins, proteoses, peptones, and polypeptides.

3. *Uric acid determination.*—Groups of three dried insects at each stage were pulverized in a mortar and made up into a suspension in 5 per cent sodium carbonate solution. This was then brought slowly to a boil in a small Erlenmeyer flask and, when cool, was centrifuged to throw down the insoluble residue. The supernatant solution was collected in a 100-ml. volumetric flask and made up to the 100-ml. mark with distilled water. A portion of this diluted solution was vacuum-filtered through a glass filter to remove cloudiness or slight discoloration which might interfere with subsequent colorimetric determinations. A 5-ml. aliquot of the filtrate was treated with tungstic acid to remove proteins and their breakdown products, and 5-ml. aliquots of the supernatant solution were analyzed in duplicate for uric acid by the Chiles technique (Gradwohl, 1943, p. 206) using a Duboscq colorimeter. Values for uric acid obtained in this way were multiplied by the appropriate dilution factors and then by 0.334, the result being uric acid nitrogen in milligrams for the three insects analyzed.

As a check on the adequacy of the fractionation method outlined above, comparisons were made between the total nitrogen values (expressed in percentage of wet weight) obtained by total analysis and the total nitrogen values arrived at by summation of the three fractions analyzed in the fractionation series. The results of this comparison are presented in Figure 1, with indications of the three stages at which there are significant differences between the values for fractionation and for total analysis. From this comparison it may be concluded that there is some loss of material in handling during the fractionation experiments but that, on the whole, the fractionation method outlined above is satisfactory for the estimation of nitrogen distribution in the insect. The greatest loss of material is probably incurred in the glass-filtration step, but for several reasons the inclusion of this operation is considered desirable.

In order to minimize the effect of possible inaccuracies in the timing of the periods during the pupal stage, the results of analyses on insects collected on each of two consecutive days were grouped together for purposes of calculation. For example, values established for pupae collected on the second and third days were calculated together under the heading, "Pupae, 2–3 days"; insects killed on the fourth and fifth days and on the sixth and seventh days of pupal life were similarly grouped.

TABLE 1

COMPARISON OF THE MEAN VALUES FOR TOTAL NITROGEN AT EARLY AND LATE STAGES IN METAMORPHOSIS, WITH THE PROBABLE SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS

Stages Compared	Total Nitrogen (Mg.)	Difference between Means	PE_{diff}	$\frac{Diff.}{PE_{diff}}$
Larva and late pupa.....	$\begin{cases}4.058 \pm 0.168 \\ 4.157 \pm 0.105\end{cases}$	0.099	0.198	0.5
Larva and adult.....	$\begin{cases}4.058 \pm 0.168 \\ 4.091 \pm 0.109\end{cases}$	0.033	0.2	0.165
Early prepupa and late pupa.....	$\begin{cases}4.440 \pm 0.171 \\ 4.157 \pm 0.105\end{cases}$	0.283	0.201	1.41
Early prepupa and adult.	$\begin{cases}4.440 \pm 0.171 \\ 4.091 \pm 0.109\end{cases}$	0.349	0.176	1.98
Late prepupa and late pupa.....	$\begin{cases}4.703 \pm 0.22 \\ 4.157 \pm 0.105\end{cases}$	0.546	0.244	2.24
Late prepupa and adult..	$\begin{cases}4.703 \pm 0.22 \\ 4.091 \pm 0.109\end{cases}$	0.612	0.282	2.17
Early pupa and late pupa.	$\begin{cases}4.208 \pm 0.102 \\ 4.157 \pm 0.105\end{cases}$	0.051	0.146	0.35
Early pupa and adult....	$\begin{cases}4.208 \pm 0.102 \\ 4.091 \pm 0.109\end{cases}$	0.117	0.204	0.573

RESULTS

Table 1 presents a comparison of the mean values for total nitrogen, obtained by total analyses performed individually on ten insects at each immature stage listed and on twenty adults. These figures show that there is no gain or loss of nitrogen during metamorphosis, no significant differences appearing between amounts of nitrogen determined at early and at late stages in metamorphosis.

Figure 2 charts the course of changes in the distribution of nitrogen between the fractions studied. These values are plotted as percentages of the total nitrogen, in order to make them consistently comparable; the changes in absolute values in milligrams give a somewhat confused picture, owing to large variation in the mean weights of insects analyzed at various stages, while figures expressed as percentages of wet weights reflect the differences in water content shown at different periods during metamorphosis.

The values in Figure 2 for Fraction C represent the nitrogen present in all compounds soluble in water but not precipitable by tungstic acid. The major portion of this fraction is probably com-

posed of amino acids and urates. This fraction, then, contains not only substances available to the insect for the construction of definitive tissue but also compounds in which excretory nitrogen is stored for subsequent elimination. This curve is broken down into its two major components in a later figure (see Fig. 3).

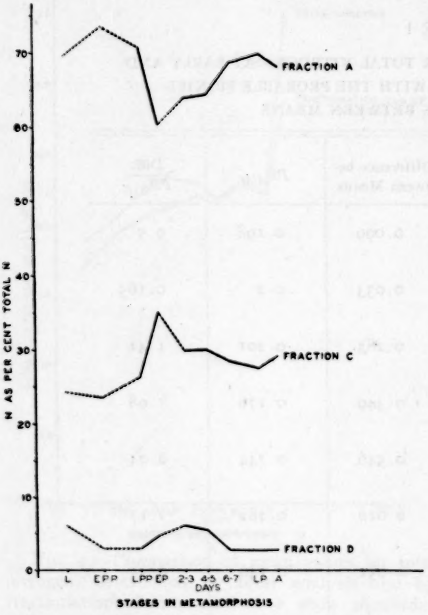

FIG. 2.—Fluctuations of nitrogen in fractions *A*, *C*, and *D*, in per cent of total nitrogen, during metamorphosis. Abbreviations as in Fig. 1.

The statistical evidence for the significance of differences between values for Fractions A, C, and D at successive stages is presented in Tables 2, 3, and 4, respectively. These values are expressed in percentages of the total nitrogen present and constitute the data used in the construction of Figure 2.

Figure 3 shows curves similar to those in Figure 2, with the exception that Fraction C has been broken down into its two major constituents by the subtraction of values for uric acid nitrogen, determined in a separate analytical series, from the total nitrogen in Fraction C. To incorporate the data from the uric acid determinations into Figure 3, values for probable total nitrogen content of the insects in the uric acid series were calculated, using the total nitrogen data from the fractionation series. The values for uric acid nitrogen in milligrams were expressed as percentages of these figures for probable total nitrogen. The uric acid as per cent total nitrogen values was then substracted from the figures in the fractionation series, Fraction C as per cent total nitrogen. These two series of points, "Fraction C less uric acid" and "uric acid" were then plotted in Figure 3. Table 5 presents the data used for the incorporation of uric acid values into Figure 3. Owing to the relatively small numbers of insects used in the uric acid series, the differences between values plotted in these curves cannot be compared with one another for significance.

Removal of the uric acid nitrogen from the Fraction C values effects few changes in this curve other than reduction in percentage at each stage. The apparent (but not significant) increase in Fraction C between early and late prepupae (Fig. 2) appears to be due to a rise in the percentage of uric acid, as amino acid nitrogen remains practically unchanged. Otherwise, the nature of the curve for Fraction C less uric acid is not greatly unlike that for Fraction C as analyzed.

The curve for uric acid nitrogen begins with the early prepupal stage, as repeated attempts failed to reveal measurable quantities of this substance in the larvae analyzed. The increase in the uric acid fraction between early and late prepupal stages probably indicates that during the prepupal stage excretory material is not eliminated as it is during the larval instars.

TABLE 2

COMPARISON OF MEAN VALUES FOR FRACTION A AS PERCENTAGE OF TOTAL NITROGEN, WITH THE PROBABLE SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS, AT SUCCESSIVE STAGES DURING METAMORPHOSIS

Stages Compared	Fraction A Nitrogen, Per Cent Total Nitrogen	Difference between Means	PE_{diff}	$\frac{Diff.}{PE_{diff}}$
Larvae and early prepupae	69.57 ± 1.279 73.51 ± 1.265	3.94	1.799	2.19
Early and late prepupae	73.51 ± 1.265 70.68 ± 0.4	2.83	1.326	2.13
Late prepupae, early pupae	70.68 ± 0.4 60.31 ± 0.93	10.37	1.012	10.25
Early pupae, pupae, 2–3 days	60.31 ± 0.93 63.96 ± 0.595	3.65	1.104	3.31
Pupae, 2–3 days; pupae, 4–5 days	63.96 ± 0.595 64.44 ± 0.83	0.48	1.021	0.47
Pupae, 4–5 days; pupae, 6–7 days	64.44 ± 0.83 68.73 ± 1.119	4.29	1.39	3.09
Pupae, 6–7 days, late pupae	68.73 ± 1.119 69.82 ± 0.73	1.09	1.33	0.82
Late pupae and adults	69.82 ± 0.73 68.11 ± 0.83	1.71	1.105	1.55

TABLE 3

COMPARISON OF MEAN VALUES FOR FRACTION C AS PERCENTAGE OF TOTAL NITROGEN, WITH THE PROBABLE SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS, AT SUCCESSIVE STAGES DURING METAMORPHOSIS

Stages Compared	Fraction C Nitrogen, Per Cent Total Nitrogen	Difference between Means	PE_{diff}	$\frac{Diff.}{PE_{diff}}$
Larvae and early prepupae	24.34 ± 0.797 23.59 ± 1.124	0.75	1.38	0.53
Early and late prepupae	23.59 ± 1.124 26.35 ± 0.51	2.76	1.23	2.24
Late prepupae and early pupae	26.35 ± 0.51 35.03 ± 1.1	8.68	1.21	7.17
Early pupae and pupae, 2–3 days	35.03 ± 1.1 30.00 ± 0.584	5.03	1.24	4.06
Pupae, 2–3 days; pupae, 4–5 days	30.00 ± 0.584 30.10 ± 0.85	0.1	1.03	0.097
Pupae, 4–5 days; pupae, 6–7 days	30.10 ± 0.85 28.50 ± 0.955	1.6	1.27	1.26
Pupae, 6–7 days, late pupae	28.50 ± 0.955 27.51 ± 0.81	0.99	1.25	0.79
Late pupae and adults	27.51 ± 0.81 29.10 ± 0.822	1.59	1.15	1.38

Curves for Fractions A and D in Figure 3 are identical with those in Figure 2.

In Figures 2 and 3 the dotted lines in the curves represent periods in which the actual course of the changes is not known. Random samples of third instar larvae were used, the ages of which were uncertain. Nothing was determined as to the changes in the nitrogenous fractions during the prepupal stage, since only early and late prepupae were analyzed in these determinations. As no analyses were made on the first day following the day of pupation, the actual course of the changes in values between the day of pupation and the second day following has not been traced.

TABLE 4

COMPARISON OF MEAN VALUES FOR FRACTION D AS PERCENTAGE OF TOTAL NITROGEN, WITH THE PROBABLE SIGNIFICANCE OF DIFFERENCES BETWEEN MEANS, AT SUCCESSIVE STAGES DURING METAMORPHOSIS

Stages Compared	Fraction D Nitrogen, Per Cent Total Nitrogen	Difference between Means	PE_{diff}	$\frac{Diff.}{PE_{diff}}$
Larvae and early prepupae	6.08 ± 0.826 2.89 ± 0.42	3.19	0.93	3.44
Early and late prepupae	2.89 ± 0.42 2.97 ± 0.27	0.08	0.499	0.16
Late prepupae and early pupae	2.97 ± 0.27 4.66 ± 0.43	1.69	0.51	3.33
Early pupae and pupae, 2–3 days	4.66 ± 0.43 6.03 ± 0.36	1.37	0.56	2.45
Pupae, 2–3 days; pupae, 4–5 days	6.03 ± 0.36 5.46 ± 0.353	0.57	0.5	1.13
Pupae, 4–5 days; pupae, 6–7 days	5.46 ± 0.353 2.76 ± 0.454	2.7	0.57	4.69
Pupae, 6–7 days, late pupae	2.76 ± 0.454 2.66 ± 0.13	0.1	0.48	0.21
Late pupae and adults	2.66 ± 0.13 2.77 ± 0.204	0.11	0.24	0.45

DISCUSSION

Many authors have reported that the process of metamorphosis in holometabolous insects is accompanied by no significant loss of nitrogen (Kellner, Sako, and Sawano, 1884, and Inouye, 1912, for the silkworm; Heller, 1924, for *Deilephila;* Frew, 1929, for *Calliphora;* Evans, 1932, for *Lucilia;* and others). Table 1 shows that this is true also of the Japanese beetle. Certain aspects of this are surprising; for example, at pupation the larval skin is shed by the insect, and, upon emergence of the adult, the pupal skin is cast. Both these skins may be shown to contain nitrogen. In addition, it is commonly held that the adult, shortly after emergence, rids itself of nitrogenous wastes accumulated during the pupal period. Yet comparisons between prepupae and pupae, pupae and adults, or even larvae and adults show no significant differences in the amounts of nitrogen present. However, the num-

bers of individuals analyzed in these investigations are comparatively small; it is possible that the expected differences would materialize if enough individuals were subjected to analysis.

An examination of the data presented in Figure 2 reveals that the fraction containing chitin nitrogen and insoluble protein nitrogen reaches its maximum value in percentage of the total nitrogen when the mature larva becomes an early prepupa. This point also marks the lowest level of the soluble protein and peptone nitrogen, probably indicating that at this time the construction of the larval insect has been completed. A similar conclusion was drawn by Evans (1932) for the development of *Lucilia*, in which the highest value for insoluble protein nitrogen was found to occur on the second day after the cessation of feeding and to coincide with the lowest point in the curve of soluble protein nitrogen changes. In the Japanese beetle the prepupal stage is not marked by any significant variations between the three fractions under consideration, although gross external signs indicate that histolysis has begun; the appendages have begun to degenerate, and the body has become shrunken within the larval skin (Ludwig, 1931). Pupation is accompanied by a relatively tremendous change in nitrogen distribution, Fraction A undergoing a marked decrease, while Fractions C and D show significant increases. While in these determinations no attempt has been made to effect a separation of values for chitin nitrogen and insoluble protein nitrogen in Fraction A, figures previously published for other insects (e.g., Kellner, Sako, and Sawano, 1884; data cited above) indicate that the amount of chitin nitrogen is relatively small. The major factor in the decrease in Fraction A values may then be taken as the breakdown of the proteins of the late prepupa into simpler compounds, which appear in Fractions C and D and bring about their conspicuous increases. By the second day following pupation, Fractions A and C show changes in the reverse directions, while the soluble protein and peptone nitrogen values do not change significantly. The remainder of the pupal period is characterized by a gradual re-

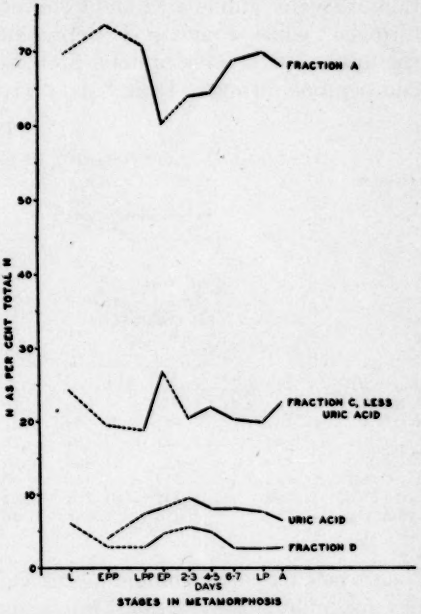

FIG. 3.—Fluctuations of nitrogen in fraction *A*, fraction *C* less uric acid, uric acid, and fraction *D*, in per cent of total nitrogen, during metamorphosis. Abbreviations as in Fig. 1.

turn of the values for the three fractions to the approximate levels found in the late prepupa. The interval between the fourth and seventh days of pupal life is marked by considerable complementary shifts between Fractions A and D, probably indictaing that chitin and insoluble protein are being rapidly synthesized from soluble proteins, proteoses, and peptones. Fraction C during this period remains relatively constant in its proportion to the total nitrogen.

Evans (1932) analyzed the nitrogenous components of *Lucilia* during metamorphosis, recovering these substances as chitin, insoluble protein, soluble protein, proteoses and peptones, amino acids, and uric acid and determining the nitrogen present in each of these fractions. Fraction C in Figure 2 thus corresponds approximately to a combination of Evans' amino acid and excretory nitrogen, while Fraction D represents the total of his soluble protein, proteose, and peptone nitrogen. Evans' data have been recalculated by combining his values for individual fractions into more inclusive groupings which are roughly equivalent to those dealt with in the present studies, and by expressing these combined figures in percentage of the total nitrogen at each stage. Treated in this manner, the variations in the chitin and insoluble protein values for nitrogen in *Lucilia* exhibit an initial increase in the late larval stage, followed by a gradual decrease from their highest figure, 60.5 per cent, to their lowest point, 47.3 per cent, which marks the third day following the day of pupation. A large increase begins immediately, reaching 69.3 per cent on the fifth day and 72 per cent on the eighth day. This is the maximum value for the pupal period, the ninth and last day showing a drop to 68.7 per cent. Reference to Figure 2 shows that these variations follow the same general pattern as those found in the Japanese beetle. There are, of course, differences which probably reflect fundamental dissimilarities in developmental physiology between the two insects; for example, in *Lucilia* the minimum value for the insoluble fraction is found on the third day following pupation, while in *Popillia* it occurs in the fresh pupa.

TABLE 5

DATA USED IN INCORPORATION OF URIC ACID NITROGEN VALUES INTO FIGURE 3

Stages	Fractionation Series				Uric Acid Series				C, Per Cent Total Nitrogen Less Uric Acid Nitrogen, Per Cent Total Nitrogen
	Mean Wet Wt.	Total Nitrogen Per Cent Wet Wt.	Fraction C (Mg.)	C, Per Cent Total Nitrogen	Mean Wet Wt.	Probable Total Nitrogen	Uric Acid Nitrogen	Per Cent Probable Total Nitrogen	
Early PP...	192.3	1.95	0.901	23.59	199.5	3.89	0.159	4.09	19.5
Late PP....	182.3	2.092	1.003	26.35	192.4	4.025	0.301	7.84	18.87
Early P....	187.3	2.105	1.364	35.03	201.6	4.244	0.349	8.22	26.81
P., 2–3.....	207.8	2.211	1.381	30.00	173.8	3.843	0.367	9.55	20.45
P., 4–5.....	212.3	2.15	1.349	30.10	205.5	4.418	0.359	8.12	21.98
P., 6–7.....	187.8	2.292	1.198	28.5	169.5	3.884	0.317	8.16	20.34
Late P.....	190.9	2.227	1.162	27.51	194.3	4.327	0.332	7.67	19.84
Adults.....	123.2	2.918	1.040	29.10	113.4	3.309	0.216	6.53	22.57

As shown in Figure 2, the course of changes in Fraction C in the Japanese beetle increases from a minimum value of 23.5 per cent in the early prepupa to a maximum of 35 per cent at pupation, subsequently showing a decrease by the third day to 30 per cent and falling gradually to 27.5 per cent by the end of the pupal period. In *Lucilia* the combined amino acid and excretory nitrogen values decrease initially from 7.4 in the mature larva to 5.6 per cent in the prepupa (nonfeeding larva) and then show a gradual and sustained increase to a maximum of slightly over 13 per cent by the end of pupal life. If Evans' data for amino acid and excretory nitrogen in *Lucilia* are now expressed separately

as percentages of the total nitrogen, the amino acid nitrogen in this insect is observed to remain practically constant throughout metamorphosis. Values for this fraction decrease from 3.7 per cent in the larva to 2.1 per cent in the prepupa, rise to 2.5 per cent by the time of pupation, and, by the last day of pupal life, have fallen only to 1.6 per cent. The figures for amino acid nitrogen in the Japanese beetle, presented in Figure 3, indicate that these values in percentage of the total nitrogen show a considerable rise at the time of pupation but otherwise do not change materially. The level to which the amino acid nitrogen values return after the rise at pupation is somewhat higher than that found in the late prepupa.

The large difference in size between the amino acid and excretory nitrogen fractions in *Lucilia* and those in *Popillia* may be due to genetic differences between the two forms, although another explanation might be the differences in analytical technique used by Evans and in the present work. In Evans' work the excretory nitrogen was determined (presumably) by the Kjeldahl method, while amino acid nitrogen was determined by the Sørensen formol titration technique. Admittedly, also, the difference between Fraction C nitrogen and uric acid nitrogen in the present studies may include more than amino acid nitrogen.

Heller (1924) studied the changes in the nitrogenous substances in *Deilephila euphorbiae* during the pupal period. He found that amino acid nitrogen increased from 28.9 per cent of the total nitrogen on the day of pupation to a maximum of 32 per cent on the 6th day, when tissue disintegration had begun. By the time the complete outline of the developing moth could be distinguished, subsequent to the 191st day, the amino acid nitrogen had decreased to 17.2 per cent of the total nitrogen; at emergence, on the 273d day, it constituted only 11.8 per cent of the total nitrogen.

From figures published by Courtois-Drilhon (1931) it is possible to calculate values for amino acid nitrogen as percentage of total nitrogen during metamorphosis of the moths she studied. In *Attacus pernyi* the monthly figures during the last six months of pupal life are as follows: 8.96, 8.92, 9.31, 10.54, 11.75, and 6.55 per cent of total nitrogen. The value in the adult of this species is 3.64 per cent. Other species of moths show comparable results.

As shown in Figure 3, the results of the present investigations indicate that the fluctuations in amino acid nitrogen in *Popillia* generally follow a pattern characteristic of other holometabolous insects for which information is available. Although the changes in different insects are found at different times relative to the total duration of the pupal stage, the majority of the results available show a rise in the amino acid fraction at some time during pupal life and a subsequent decline in this fraction. *Lucilia* is exceptional in this respect, no marked changes in the amino acid fraction having been detected.

Considering, now, the variations in Fraction D, Figure 2 shows that in *Popillia* the nitrogen in the tungstic acid precipitate falls from the larval value of 6.1 to 2.9 per cent, which is the level maintained through the prepupal period. A slow increase then begins, which reaches its maximum by the third day after pupation, at which time the Fraction A and C values have begun to return to their former levels following the changes accompanying pupation. The original prepupal low level is reached again by Fraction D on the seventh day and is maintained through emergence. In *Lucilia* the changes in the correspond-

ing combined fraction (soluble protein, proteose, and peptone nitrogen) are somewhat different. Here the values show an initial decrease from 37.3 to 33.8 per cent, followed by a gradual increase to a maximum of 40.5 per cent by the third day after pupation. A sudden decrease to 17.7 per cent is noted by the fifth day; the value for the eighth day is 14.7 per cent; and for the last day of pupal life it is 18.1 per cent. Evans' separation of soluble from insoluble substances in *Lucilia* was effected by filtration on a Buchner funnel; in the present studies this separation was performed by centrifugation followed by vacuum filtration through a glass filter (Jena, *5 auf 3 Grad;* mean pore diameter 1.01–1.09 μ; Porter, 1946). Possibly as a result of this difference in analytical procedure, the proportion of chitin nitrogen and insoluble protein nitrogen is somewhat lower in *Lucilia* than the present determinations indicate for *Popillia*, while the reverse is true of the percentages of soluble protein, proteose, and peptone nitrogen.

Unfortunately, the data presented by Evans (1934) from detailed analyses on *Tenebrio molitor* cannot be directly compared with the results of the present studies on *Popillia*. In general terms, however, in the meal worm he found no great changes other than in uric acid nitrogen during the course of metamorphosis. In Evans' words:

> Insoluble protein, representing the amount of organised tissue present, falls slowly until the ninth day from the institution of metamorphosis. Soluble protein also decreased until the seventh day, when there is a slight rise followed by a further fall. The final fall in soluble protein is accompanied by an increase in insoluble protein preparatory to emergence. Amino-acids and proteoses remain fairly constant, the latter increasing somewhat on the ninth day during the final stages of histogenesis.

Evans surmised that the great differences between these relatively slight changes and the larger variations shown by his work on *Lucilia* reflect the fact that in the Coleoptera histolysis and histogenesis are not nearly so well marked during metamorphosis as in the higher Diptera. If this is the case, it appears that these two processes are carried on to a marked degree in the Japanese beetle; the variations in the nitrogenous components of this insect resemble those occurring in *Lucilia* more closely than they resemble those found in *Tenebrio*.

According to Weismann (1864), insect larvae at hatching are endowed with two kinds of tissues—larval and imaginal. The larval tissues are active or dominant as long as they are functional, while the imaginal tissues remain dormant until the disintegration of the larval tissues (histolysis) creates conditions favorable for their growth (histogenesis) and furnishes the necessary nutrient materials. In a series of determinations of the respiratory exchange in insects during metamorphosis, Fink (1925) established a relationship between the marked histolysis occurring in the prepupal period and the considerable lowering of the CO_2 output of the insect at this time. This decrease in the CO_2 output is accompanied by a fall in the oxygen consumption; the low level of respiratory exchange is maintained for a short time, and then a gradual increase occurs before the end of the pupal period. According to Fink, the general rising of the curve after the low phase accompanies the growth of the imaginal buds. There has been a general acceptance of this identification of histolysis and histogenesis by the fall and rise of respiratory metabolism, since it is known that these processes occur in metamorphosis,

and many authors have confirmed the existence of the "U-shaped curve" of respiratory metabolism during this period.

In the absence of histological data for the chronology of the histolytic and histogenic changes occurring in the metamorphosis of the Japanese beetle, it may be permissible to assume that the progress of these changes may be measured by the relative abundance of insoluble and soluble nitrogenous tissue components in the insect. If this is valid and if the curve of oxygen consumption may also be used as an indication of the structural changes occurring, then one might expect the drop in the value for oxygen consumption early in metamorphosis to coincide chronologically with the large complementary shift between the insoluble and the soluble nitrogenous fractions. Figure 4 compares the results of the present analytical studies with the results of studies by Ludwig (1931) on oxygen consumption in the Japanese beetle during metamorphosis. For purposes of this comparison the curves showing the fluctuations in Fractions A, C, and D have been prepared from data determined by analysis of insects on separate days rather than from combined values for two successive days, as in previous figures. Owing to the small number of insects analyzed on each day, differences between values during the pupal stage cannot be statistically compared for significance. It will be noted that the marked decrease in oxygen consumption was found to occur between the early and the late prepupa, while the shift between Fractions A and C does not take place until the formation of the pupa. Further, on the second day after pupation, when the insoluble protein and chitin fraction has begun to be reconstituted at the expense of the soluble compounds, the curve of oxygen consumption has fallen to a new low value. The gradual rise in the oxygen consumption curve during the remainder of the pupal period does, however, coincide with the synthetic activities which are completed in the late pupa. There is no marked change in the proportions of the

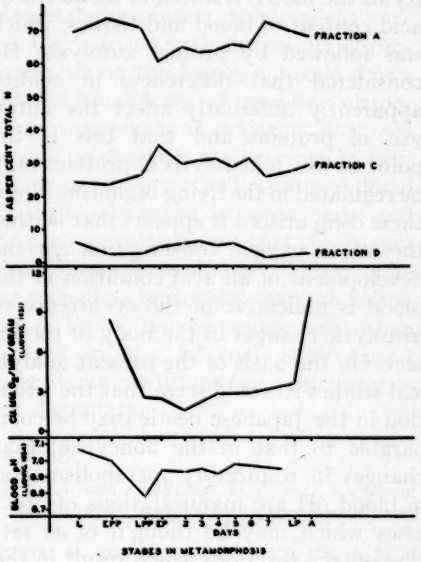

FIG. 4.—Fluctuations of nitrogen in fractions *A*, *C*, and *D*, in per cent of total nitrogen, during metamorphosis, compared with changes in respiratory metabolism and in blood pH. Abbreviations as in Fig. 1.

three fractions to correspond to the large increase in oxygen-consumption values to the level characteristic of the adult.

On the same chronological scale, Figure 4 presents for comparison the results of Ludwig's studies (1934) on changes in blood pH in the Japanese beetle during metamorphosis. Ludwig's figures show that in *Popillia* during the prepupal period there is a significant change in blood pH from a slightly alkaline to a some-

what acid condition. This development coincides with the fall in the oxygen-consumption curve but precedes the changes in the nitrogenous fractions. Bishop (1923) found that in the larva of the honeybee at the beginning of pupation, a decrease in respiratory metabolism, followed by a lowered buffer capacity in the blood, resulted in an increased acid content of blood and tissues, which was followed by protein autolysis. He considered that differences in acidity apparently differently affect the autolysis of proteins and that this is the point at which histolysis of proteins may be regulated in the living organism. From these comparisons it appears that neither the fall in oxygen consumption nor the development of an acid condition in the blood is indicative of the occurrence of histolytic changes in the body of the insect. On the basis of the present analytical studies it would seem that the situation in the Japanese beetle may be comparable to that in the honeybee; that changes in respiratory metabolism and in blood pH are manifestations of processes which may be thought of as setting off the reactions which result in the breakdown of tissue proteins into soluble degradation products.

SUMMARY

Individual Japanese beetles (*P. japonica* Newman) were killed, desiccated, and subjected to total nitrogen analysis as mature larvae, at intervals during the prepupal and pupal stages, and as adults. A parallel series was analyzed by a fractionation method in which the nitrogenous substances were recovered in three fractions: A, containing chitin nitrogen and insoluble protein nitrogen; C, containing the nitrogen of amino acids and soluble excretory products; and D, containing soluble protein, proteose, and peptone nitrogen. (Fraction B, containing all water-soluble nitrogenous substances, was separated by further analysis into Fractions C and D.) Another series of insects was analyzed for uric acid, and the uric acid nitrogen found in these was subtracted from Fraction C nitrogen, the difference being taken as amino acid nitrogen. For insects at each stage analyzed, the four values thus obtained were expressed as percentages of the total nitrogen, and their fluctuations during the period of metamorphosis were charted. The results noted are as follows:

1. No significant loss of nitrogen occurs during the course of metamorphosis. Absolute values in milligrams for the total nitrogen present at early and at late stages are not statistically different from each other.

2. In percentage of the total nitrogen, the nitrogen in water-insoluble compounds does not change between larval and late prepupal stages. At pupation there is a sudden large decrease in the proportionate size of this fraction, followed by gradual increases during subsequent days of pupal life. The late pupa contains almost the same proportion of insoluble nitrogen as does the late prepupa; there is no significant change upon emergence.

3. Nitrogen in amino acids does not change, in proportion to the total nitrogen, during late larval and prepupal life. An increase in the proportion of amino acid nitrogen occurs at pupation and is followed immediately by a decrease, which becomes more gradual during the remainder of pupal life. No significant change occurs upon emergence.

4. No uric acid could be detected in the mature larvae analyzed. In the early prepupa, uric acid nitrogen constitutes about 4 per cent of the total nitrogen; between the second and third days fol-

lowing pupation, this fraction reaches its peak of 9.5 per cent. Slight decreases apparent on subsequent days may or may not be significant.

5. The nitrogen present in Fraction D (soluble protein, proteose, and peptone nitrogen) decreases significantly between larval and early prepupal stages. It shows no change in the late prepupa but subsequently rises to a maximum percentage of the total nitrogen on the second to third day of the pupal period. By the sixth to seventh day it has returned to its former level and maintains this through emergence.

6. These complementary shifts between the nitrogenous fractions may be interpreted as indicative of the destruction of mature larval tissues at pupation, followed by gradual construction, from the materials thus made available, of the definitive structures of the adult.

7. The changes in the nitrogenous components of *Popillia* during metamorphosis are comparable in some degree with those reported from other species belonging to various insect orders. There are specific differences in the extent and chronology of the changes in the different insects for which data are available; however, the variations between the nitrogenous substances in *Popillia* follow the general pattern characteristic of holometabolous insect development.

8. Comparison with the results of previous work on the Japanese beetle shows that the breakdown of insoluble nitrogenous compounds at pupation does not coincide with either the decrease in oxygen consumption or the lowering of blood pH known to occur between early and late prepupal stages, but that this breakdown follows these changes. This may be interpreted as indicating that the histolysis of larval tissues begins only when changes in the internal environment bring about conditions favorable for protein degradation.

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RELATION BETWEEN LIPID CONTENT OF CUTICLE, DURATION OF DIAPAUSE, AND RESISTANCE TO DESICCATION OF PUPAE OF THE CYNTHIA MOTH

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THE pupae of the Cynthia moth, *Samia walkeri*, are very resistant to desiccation. Ludwig (1943) found that overwintering pupae are able to survive more than 90 days at 25° C. and 0 per cent relative humidity, many of the exposed pupae producing normal moths. Under these conditions there is a gradual loss of weight throughout pupal diapause, amounting to less than 5 per cent of the original weight. Following diapause, the initiation of development is accompanied by a more rapid loss of weight, which continues until adult emergence. The pupae of the Promethea moth, *Callosamia promethea*, are also very resistant to desiccation during diapause (Ludwig, 1946). When the pupae are placed at different temperatures over anhydrous $CaCl_2$, the loss of weight is very gradual and appears to occur at the same rate regardless of the saturation deficiency of the atmosphere. However, when Promethea pupae are treated daily with peanut oil, the loss of weight over anhydrous $CaCl_2$ is very rapid and is then approximately proportional to the saturation deficiency. It was postulated that the oil increased the rate of water loss from the pupae by dissolving a fatty or waxy substance from the surface of the cuticle.

Within recent years many investigators have been interested in the elimination of insect diapause by experimental procedures. In many cases exposure of the diapausing stages to low temperature has been effective (Bodine, 1925, 1932; Williams, 1946). Pepper (1937) succeeded in breaking the larval diapause of the sugar-beet webworm, *Loxostege sticticales*, by treating the larvae with xylol and other fat solvents. He believed that the chemicals brought about their effect by the activation of lipolytic enzymes. Slifer (1946) was able to break the diapause of the egg of the grasshopper, *Melanoplus differentialis*, with various fat solvents. She believed that the fat solvent dissolved a waxy plug from the hydropyle and thus permitted the intake of the water needed for further development.

In view of these experiments it was decided to treat pupae of the Cynthia moth with ether or with peanut oil to determine whether the removal of the lipids from the cuticle would have any effect on the duration of diapause. The experiments were extended to include the effects of ether treatments on the rates of dehydration when pupae were kept at different relative humidities.

MATERIAL AND METHODS

Cynthia cocoons were collected in New York City. When brought into the laboratory, the pupae were removed from

their cocoons and kept at room temperature until needed. The general procedure followed throughout these experiments was as follows: the pupae were divided into groups of ten, treated with a fat solvent (peanut oil or ether), weighed on a Chainomatic balance, transferred to desiccators at the desired humidity, and kept in incubators at 25° C. Control groups were placed at 0, 56, 76, and 100 per cent relative humidities. These humidity values were obtained by using chemicals in the bases of ordinary desiccators as follows: for 0 per cent, anhydrous $CaCl_2$; for 56 per cent, a saturated solution of NaBr; for 76 per cent, a saturated solution of NaCl; and for 100 per cent, distilled water. Pupae of these control groups were weighed weekly.

To determine whether the removal of the lipids from the cuticle has any effect on the duration of pupal diapause, groups of pupae were treated with peanut oil or immersed in absolute ethyl ether for 5 minutes and placed in desiccators over distilled water. Peanut-oil treatments consisted of rolling the pupae on filter paper moistened with peanut oil until the cuticle was thoroughly coated with the oil. At the end of 10 minutes the oil was cleaned from the surface, and the pupae were weighed and placed in desiccators at 100 per cent relative humidity. Pupae of these two groups were weighed twice weekly until they were killed by molds or until adult emergence. Several hundred pupae were immersed in ether for 5 minutes and then kept over moist cotton at 25° C. until death or until adult emergence. The duration of the pupal stage in these treated pupae was compared with its duration in normal insects.

For purposes of comparison with results previously obtained with Promethea pupae (Ludwig, 1946), groups of Cynthia pupae were given daily peanut-oil treatments and placed at 0, 56, and 76 per cent relative humidities. These pupae were weighed daily until constant weight readings were obtained.

To study the relation between the lipid content of the cuticle and resistance to desiccation, pupae were immersed in ether for varying periods of time. Following this treatment, they were allowed to stand at room temperature in open vials for from 1 to 2 hours to insure the evaporation of any ether which might have been absorbed. They were then weighed and placed in desiccators at the desired humidity. Groups of pupae were immersed in ether for 5 minutes and placed at 0, 56, and 76 per cent relative humidities. Others were immersed for 30 seconds and for 1 and 2 minutes in ether and kept over anhydrous $CaCl_2$. Others were immersed daily in ether for 5 minutes or for 2 minutes and placed over anhydrous $CaCl_2$. The pupae of these groups were weighed daily for a 42-day period or until constant weight readings were obtained.

The amount of lipid removed by the above treatment was determined by filtering the ether, in which the pupae had been immersed, through a Soxhlet thimble into clean weighed beakers. The beakers were covered with Petri dishes to keep out dust and were allowed to stand at room temperature until their weights remained constant. The weight of the lipid was obtained by subtracting the original weights of the beakers from their final weights.

OBSERVATIONS

Pupae treated with peanut oil or with ether were found to lose water very rapidly, except in saturated air. However, at this high humidity, they were subject to attacks by parasitic fungi which grew from the spiracles and the sutures of the exoskeleton. As a result, many of the exposed pupae did not survive through

metamorphosis even at high humidities. However, a large number of moths did emerge from the ether-treated pupae, and they appeared normal except that the wings were not inflated properly. In no case was there any evidence of a shortened diapause as a result of peanut-oil or ether treatments, the moths from experimental and normal pupae emerging simultaneously.

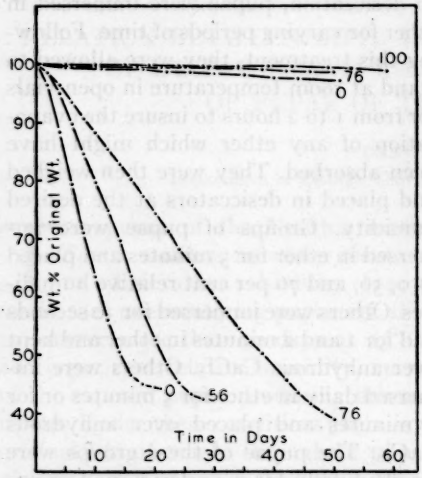

FIG. 1.—Rate of desiccation of diapausing pupae at different relative humidities. *Upper graphs*, normal pupae; *lower graphs*, pupae given daily treatments with peanut oil. Numbers indicate relative humidities.

The upper graph in Figure 1 shows the rates of weight loss of normal pupae at different humidities. This loss was very gradual, regardless of humidity, the total loss in dry air averaging only 3 per cent of the original weight during a 50-day period. The lower graphs of this figure show the rates of weight loss of pupae given daily treatments with peanut oil at the relative humidities of 0, 56, and 76 per cent. The loss of weight is more rapid, the lower the relative humidity, and it is roughly proportional to the saturation deficiency of the air. The final weights shown in the figure do not represent the dry weights of the pupae, since a considerable amount of oil was absorbed by the tissues of the treated insects.

Cynthia pupae are very resistant to immersion in ether, many surviving more than five or six consecutive daily 5-minute exposures. The effects of a single 5-minute immersion in ether on the rate of dehydration at different humidities are shown in Figure 2. In this case the rate of weight loss is also greater, the lower the humidity; and the agreement with the "saturation deficiency law" (Mellanby, 1935) is much better than with the peanut-oil-treated pupae. The final weight, 25.3 per cent of the original value (shown in Fig. 2), is believed to represent the dry weight of the pupae.

In Figure 3 are plotted the rates of weight loss at 0 per cent humidity for pupae immersed in ether for different periods of time. The figure shows that the longer the period of immersion, the greater the rate of dehydration. These results indicate that longer exposures to

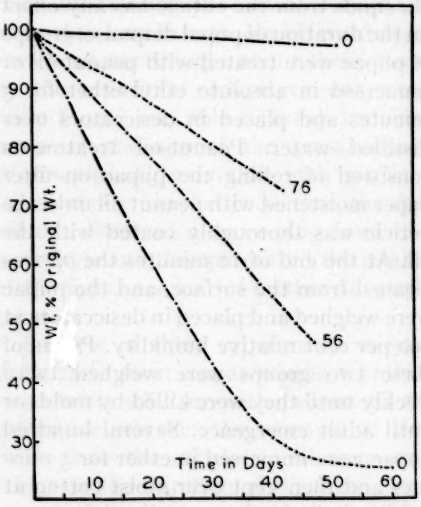

FIG. 2.—Rate of desiccation of pupae at different relative humidities following a single 5-minute immersion in ether. Numbers indicate relative humidities. *Upper line*, normal pupae.

ether remove more lipid from the cuticle and thus make it more permeable to water. Table 1 shows that, in general, the amount of lipid removed is greater, the longer the period of immersion. Pupae immersed in ether for only 30 seconds responded in two ways: five of the ten pupae lost weight at a constant rate throughout the experiment; the other five lost weight at a much slower rate during the first week, after which the rate was further decreased and remained low until the end of diapause, when it again increased. This observation suggests that, following a very brief exposure to ether, the film of lipid is at least partly restored by a few pupae. Figure 3 also contains graphs showing the rates of weight loss when pupae were immersed daily in ether for 2 and for 5 minutes. These pupae lost weight more rapidly than those given a single exposure for the same length of time, indicating that not all the lipid is removed in a single treatment.

TABLE 1

AMOUNT OF LIPID REMOVED FROM CYNTHIA PUPAE BY IMMERSION IN ETHER

No. of Pupae	Time Immersed in Ether	Weight of Lipid per Pupa (Mg.)
81.....	5 minutes	1.26
40.....	2 minutes	0.82
10.....	1 minute	0.85
10.....	30 seconds	0.56

During the course of these experiments, a few moths emerged from ether-treated pupae at humidities of 56 and 76 per cent. Figure 4 contains a comparison of the rates of weight loss of normal pupae and of these ether-treated pupae during the period preceding adult emergence. The graphs for normal pupae were obtained from the work of Ludwig (1943). The loss of weight of ether-treated pupae continued constant until emergence, whereas the loss of weight of normal pupae was very slow during diapause and much more rapid during the 2 weeks preceding adult emergence. A possible explanation for this difference might be that during normal metamorphosis the lipid content of the cuticle is reduced, thus permitting a more rapid loss of water toward the end of the metamorphosing period, whereas exposures to ether removed this substance from the experimental animals. To test this hypothesis, pupae were allowed to develop until 1 or 2 days before adults emergence (as evidenced by the coloring of the body and the wing pads); they were then immersed in ether for 5 minutes, and the

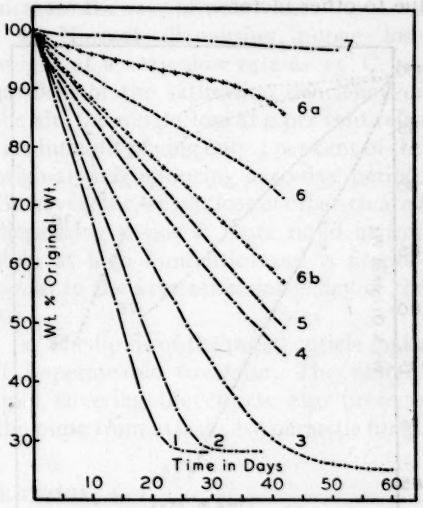

FIG. 3.—Rate of desiccation of pupae over anhydrous $CaCL_2$ following immersion in ether for different periods of time. Curve *1*, pupae immersed 5 minutes daily; curve *2*, pupae immersed 2 minutes daily; curve *3*, pupae immersed a single 5-minute period; curve *4*, pupae immersed a single 2-minute period; curve *5*, pupae immersed a single 1-minute period; curve *6*, pupae immersed a single 30-second period; curve *6a*, pupae immersed a single 30-second period but which restored the lipid film; curve *6b*, pupae immersed a single 30-second period but which did not restore the lipid film; curve *7*, normal pupae.

amount of lipid removed by this treatment was determined. This experiment showed that there is no decrease in the amount of lipid present on the cuticle of metamorphosing individuals. The increase in the rate of water loss accompanying the development of adult structures in normal pupae must therefore be due to other factors.

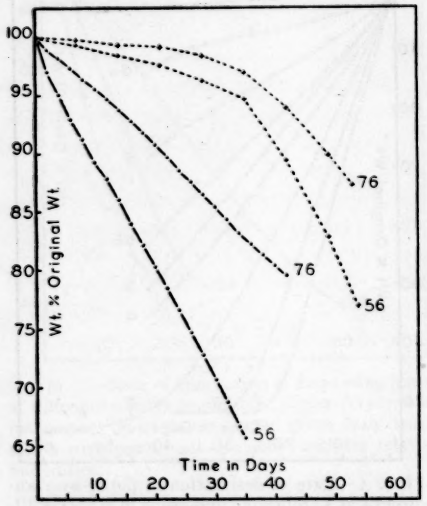

FIG. 4.—Rate of desiccation of normal and ether-treated pupae during time just preceding adult emergence. *More broken lines*, normal pupae; *more solid lines*, ether-treated pupae. Numbers indicate relative humidities.

DISCUSSION

These experiments indicate that, although treating Cynthia pupae with fat solvents renders the cuticle freely permeable to water, it does not affect the duration of diapause. In this species the termination of diapause is probably controlled by a growth-activating substance produced by the brain, as suggested by Williams (1946) for the Cecropia moth, *Platysamia cecropia*. Williams found that exposing pupae to cold stimulated the brain to produce its activating substance. He found that the chilled brain was non-specific in its effect and that chilled brains of Cynthia pupae were able to terminate pupal diapause in related species, including the Cecropia moth.

Pupae of the Cynthia moth are similar to those of the Promethea moth, *Callosamia promethea*, in their resistance to desiccation. This similarity may be correlated with the fact that the cocoons of both species are suspended from the branches of trees and are thus subjected to the drying effects of the atmosphere. A comparison of Figure 1 with Ludwig's Figures 1 and 2 (1946) shows that in both species exposure to peanut oil removes a fat-soluble substance from the cuticle and thus renders it more permeable to water.

The observations that the amount of lipid removed from the cuticle and the rate of dehydration at low humidities are dependent on the length of time that pupae are immersed in ether suggest that, normally, pupae do not lose water through the cuticle because the lipid present makes it impermeable to water. The small amount of weight lost when diapausing pupae are placed in dry air must then be the loss of dry material through oxidation and the loss of some water through the spiracles. This suggestion is in accord with the conclusion of Mellanby (1934) that practically all the water lost by an insect evaporates through the tracheal system, the cuticle being practically impermeable to water. The greater rate of weight loss associated with the development of adult structures in normal pupae is probably the result of an increased rate of metabolism, with greater production of metabolic water. The increase in metabolic rate also results in an increase in tracheal ventilation, thus permitting a greater loss of water from the developing insect. In ether-treated pupae no increase in the rate of weight loss is evident following diapause, since, in these insects, the cu-

ticle is water-permeable. Hence in treated pupae the rate of dehydration is not necessarily associated with tracheal ventilation.

The high mortality of ether-treated pupae due to attacks of parasitic fungi suggests that the lipids of the cuticle performs two functions: first, they make the cuticle impermeable to water; and, second, the film of lipid covering the cuticle serves as a protection against fungal attacks, since normal pupae are not subject to these attacks.

At present nothing is known regarding the chemical nature of this lipid. However, preliminary melting-point determinations indicate that it is not a pure compound but is probably a mixture of fatty and waxy substances.

SUMMARY

1. Removal of lipids from the cuticle of diapausing Cynthia pupae by peanut-oil or ether treatments increases its permeability to water but has no effect on the duration of diapause.

2. The amount of lipid removed from the cuticle and the rate of dehydration over anhydrous $CaCl_2$ are dependent on the length of time that the pupae are immersed in ether. Long exposures remove more lipid than short exposures do and increase the rate of water loss.

3. Normal diapausing pupae lose weight at a very slow rate at 25° C. regardless of the saturation deficiency of the air, the weight loss at 0 per cent relative humidity being only 3 per cent of the original weight during a 50-day period. However, the weight loss of ether-treated diapausing pupae is more rapid at low than at high humidities and is proportional to the saturation deficiency of the air.

4. The lipids of the pupal cuticle make it impermeable to water. The film of lipid covering the cuticle also protects the pupa from attacks by parasitic fungi.

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TOXICITY AND PERMEABILITY[1]

II. THE ENTRANCE OF ARSENIC INTO LARVAE OF THE FLESH FLY, SARCOPHAGA SECURIFERA, VILL.

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INTRODUCTION

In the first paper of this series (Hoskins, 1932) data were given to support the conclusion that acidic solutions of trivalent arsenic (pH 5) cause mortality of mosquito pupae approximately four times as rapidly as do basic solutions (pH 11) of the same strength. From this it was deduced that arsenious acid molecules penetrate the integument more readily than do arsenite ions. However, toxicity data are a somewhat uncertain measure of concentrations, especially when the latter vary with time; and hence a more direct determination of the penetration of insect integument was desirable in attempting to evaluate the roles of molecules and ions of soluble insecticides.

A step in this direction was taken by Alexandrov (1935), who cut off the opposite ends of larvae of *Chironomus plumosus* and other dipterous species and squeezed out the body contents. By tying one end of the isolated cuticle, he obtained a little bag into which any desired indicator could be placed. The other end was then tied and the cuticle and contents immersed in solutions of acids or bases. The relative rates of penetration of strong and weak acids and bases are shown by the periods before the indicators changed color. Thus, upon exposure of the cuticles to acidic solutions of equal pH (2.3), bromphenol blue changed from blue to yellow in 30 seconds with acetic acid, and in about 20 minutes with hydrochloric acid. Similarly, thymol blue changed from yellow to blue in 20 seconds after immersion of the cuticle in ammonia solution of pH 10.8 but not until after several hours in the case of sodium hydroxide. Morosov (1935) obtained similar results with the integuments of *Pieris rapae* and related species, and he introduced the simpler technique of fastening the cuticle formed into a bag upon the end of a glass tube, so that solutions could be renewed or changed as desired. Each of these workers studied the relative permeability of the integuments from related species and the effects of exposure to solvents and other chemicals, but neither determined the actual rate of penetration for any substance.

EXPERIMENTAL METHODS

In the present work use was made of the large Sarcophagid fly, *Sarcophaga securifera*, Vill. This insect has the advantages of a short life-cycle and easy rearing in the laboratory throughout the year. Larvae of known age can be obtained by keeping meat, e.g., fishheads, before the gravid females so that the young, which are brought forth as larvae, are not retained for variable periods. The larvae feed for about 4 days and then

[1] Contributions from the laboratory of insect physiology and toxicology, Division of Entomology and Parasitology, University of California, Berkeley, California. The authors wish to acknowledge with appreciation the helpful suggestions made by Dr. S. C. Brooks regarding organization of this paper.

spend several days in a nonfeeding, prepupal stage. The latter instars are large, and separated cuticles of 200-sq. mm. area or more with no lateral spiracular openings can be obtained. Lastly, the larvae can live under water for several hours, so submersion has little influence on the toxicity of aqueous solutions. In the present work, separate lots of larvae were reared upon fishheads kept in gallon glass jars, every effort being made to keep availability of food comparable in all cases.

Trivalent arsenic at concentrations of 0.128 M and 0.223 M in aqueous solutions was used as the toxic agent in most tests. The pH was varied from 5 to 11.5 by use of 0.2 M phosphate buffer plus the required amount of sodium hydroxide for the higher values or hydrochloric acid for the lower ones. All pH's were checked by a glass electrode with correction in the highly alkaline solutions.

The amounts of arsenic penetrating the integument or held in it or in other parts were determined by the bromate method, modified for microquantities. The reagents used were as follows: low-arsenic nitric and sulphuric acids, standard potassium bromate solutions, 0.0004–0.00004 M (i.e., 1 cc. was equivalent to 90–9 γ As), 0.01 per cent methyl orange solution, hydrazine sulphate, sodium bromide, sodium chloride, and hydrochloric acid. The latter four materials were used in a solution of the following composition: 2 gm. hydrazine sulphate, 2 gm. sodium bromide, 40 cc. concentrated hydrochloric acid solution, 700 cc. saturated sodium chloride solution, and 160 cc. distilled water.

In brief, the procedure was as follows: The larvae (one to five in number) or other material to be analyzed were placed in a 25-cc. Kjeldahl flask and heated for a few minutes with 4 cc. nitric acid. Two cc. sulphuric acid were then added, and heating was continued on a sandbath with occasional addition of nitric acid to a total amount of 10 cc. until the sample remained water-white when brought to dense fumes of sulphur trioxide. Additional heating for 2 minutes served to remove the excess nitric acid. All arsenic, irrespective of its original condition, would now be in the pentavalent state. After cooling, 5 cc. of distilled water were added, and the solution was transferred to a 100-cc. distilling flask provided with ground-glass connections. The digestion flask was rinsed with 2–3 cc. of distilled water and with 10 cc. of the hydrazine sulphate solution, both rinsing liquids being added to the distilling flask. The side-arm was connected to an air condenser, whose tip was just below the surface of a 10-cc. volume of water in a small Erlenmeyer flask. Through the upper ground-glass joint a glass tube led to the bottom of the distilling flask. When the latter was heated carefully, the following reaction occurred: pentavalent arsenic was reduced by the hydrazine and bromide to the trivalent state, which in the strongly acid solution combined with chloride ion to form arsenious chloride, $AsCl_3$. Carbon dioxide (or nitrogen) was forced through the flask by way of the upper tube fast enough to cause steady bubbling in the Erlenmeyer flask, and thus a current of gas was furnished which aided greatly in carrying over the slightly volatile arsenious chloride and incidentally prevented bumping. Heating was continued until 12–15 cc. of liquid had distilled over. The use of an inert gas stream is of great value with the small amounts of arsenic involved, for the strong heating otherwise necessary to remove all arsenious chloride can be avoided and there is no danger of forming sulphur dioxide, which ordinarily gives rise to the chief error.

The final titration was carried out at

90–95° C., with three drops of 0.01 per cent methyl orange solution as indicator. At the end-point the red color is suddenly discharged. A reagent blank was determined at intervals and whenever a reagent was changed. It usually corresponded to about 8 γ As. Titrations of known amounts of arsenic digested with insect matter as in the ordinary tests were reproducible to approximately 0.2 cc. bromate solution, which gave an uncertainty of about 5 per cent with the smallest amounts of arsenic encountered and much less with the larger amounts.

Several methods were used in determining the penetration of arsenic through the integument. The simplest consisted in submerging larvae at the depth of about an inch in the various solutions for the desired periods, carefully washing and analyzing for contained arsenic. This procedure was of little value for the purpose in mind, for it permitted both ingestion and excretion. Ingestion could be prevented readily by tying off the head end with a thread or clamping it off by means of a thin rubber membrane. This was readily done by gently compressing a larva, e.g., by laying a book on it so that the head end protruded in an extended condition over the edge of a table. Either a loop of thread or a stretched membrane previously pricked with a pin could then be slipped over the anterior end of the larva. In a similar manner both ends of the body could be clamped off, but the rear segment is so muscular that the rubber membranes were soon cast off and only thread could be used. This treatment appeared to do little harm to the larvae, for they lived for many hours. After exposure to the arsenic solution, the ends of the body were clipped off just at the ligatures so that only the body between them was analyzed.

Separated integuments were prepared by cutting off the posterior segment and expelling the body contents by rolling from the front end with a glass rod. This method of preparing the integument leaves a thin, jelly-like layer on the inside, which can be removed more or less completely by scraping lightly or rolling more firmly. Since a few tests showed that the presence of this inner layer made little difference in the permeability of integuments to arsenic, it was not ordinarily removed. It is unlikely that the outer surface was injured by the method of preparation, for the integument was never in contact with rough or abrasive material and was not stretched or folded sharply. Two methods of mounting the integuments were used. The first consisted of fastening a piece across the end of a wide, short glass tube attached to a bulb. The integument was tied over the end by means of thread and cemented with beeswax or Metallic-X cement, which extended over the edge, leaving a circle of determinable area. The tube leading to the bulb was very short, in order to obviate the danger that slowness in diffusion might retard penetration. By the second method of mounting, the baglike integument was pulled partway over the end of a glass tube, and a little Metallic-X cement was put on both ends to aid in sealing. The two methods gave equivalent results for unit area exposed. Hence it was decided that there was no effect from slowness of diffusion of arsenic away from the integument; and the second method was adopted for most of the work, since it enabled much greater areas to be used. The area exposed was almost exactly the same whether a larva was doubly ligated or its integument was fastened to a tube as just described. In all cases the 0.2 M phosphate buffer solution at the same pH as the arsenic solution was put on the other side of the cuticle.

It was necessary to make sure that the separated integuments were free from holes and that the seals were tight. This could be done in a crude way by blowing into the tube with the integument under water, but a much more sensitive method was to measure the concentration potential set up when 0.01 N potassium chloride solution was put inside the integument and the latter was submerged in a 0.1 N solution of the same salt. Connection was made through 0.1 N calomel half-cells to a Coleman electrometer. The integuments varied considerably, but many gave from 12 to 20 mv. The tiniest hole, often not detectable in any other way, reduced the potential to zero. Hence the method was used routinely, and preparations giving no potential were rejected, or the seal was repaired. No selection, however, was made among those giving appreciable readings. Both sides were thoroughly rinsed before starting the penetration experiments.

When either intact larvae or detached integuments were used, it was necessary to determine the area available for penetration. The segments of these larvae are roughly cylindrical in shape, and the total area used may be calculated by determining the length and diameter of each segment included. It was found that the much simpler procedure of multiplying the diameter of the largest segment, d, by the distance between ligatures, l, gave a product proportional to the area calculated from individual segments. The formula used was: Area = $0.88dl$. This was checked by comparing with the areas obtained by splitting the integument and placing it under a glass plate for direct measurement. The agreement was always within 3–4 per cent. In an actual experiment thirty or more from each lot of larvae used were measured, and an average area was calculated. Care was taken to choose only larvae of closely agreeing dimensions. Since tests were conducted either with considerable numbers, e.g., thirty to fifty, or with individual larvae which were separately measured, it would appear that the error involved in determination of area was not greater than 5 per cent. Immediately after ligation the larvae squirmed so much that they could not be measured, but they were always quiet upon removal from the arsenic solutions (sometimes they were dead), and all measurements were made then. This had the further advantage that the larvae had but recently been removed from solutions of approximately the same osmotic pressure and hence were in the same state of water balance.

Since the laboratory temperature usually was within the range 20°–24° C. with no wide variation in either direction, no attempt was made to secure closer regulation during the rearing of the larvae or exposure to the arsenic solutions.

RESULTS

EFFECT OF AGE

In order to study the penetration of soluble arsenic into fly larvae under various conditions of pH, concentration, etc., it was desirable to determine the effect of the age of the larvae upon the process and, if possible, to find a stage of considerable length during which the rate of penetration did not change. Groups of fifty larvae at the desired ages were taken from fishheads or after feeding was finished, doubly ligated, and submerged in 0.223 M arsenite plus 0.2 M phosphate buffer at pH 5. The pupae were sealed at each end with a bit of Metallic-X cement. The period of exposure was 24 hours, after which time the larvae or pupae were removed, carefully washed, measured, and analyzed in groups of five as described before. The averaged results of each age, expressed

in γ As/mm²/hour, are shown in Figure 1. The indicated ages are those at the beginning of exposure to the poison. This time is chosen rather than the mid-point of the exposure period because feeding, and, presumably, growth ceased when the larvae were prepared for the test. Hence it is felt that the results are more characteristic of the integuments at the beginning of exposure. It is of interest to note that no cast skin was ever found when unligated larvae were kept in arsenic solutions. Because of the minute size of the first two instars, it was not possible to work with the larvae until they were 36 hours old. The high permeability at this age decreases very rapidly and reaches a fairly constant value for the period between about 100 and 180 hours. This interval covers the last few hours of feeding and the period when the larvae leave their food and wander about before any visible change associated with pupation occurs. Unless especially mentioned, all further experiments were made within this age interval, usually near 5 days. It will be noted that the first part of the curve in Figure 1 indicates two rather abrupt changes in permeability. These may be connected with the two larval molts occurring in the interval concerned, but no study of the matter was made. The high permeability prevailing soon after pupation drops rapidly but was not investigated after the age of 326 hours.

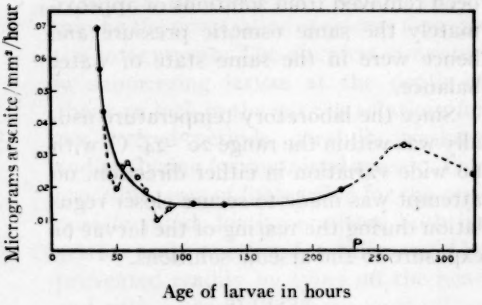

FIG. 1—Effect of age of larvae upon rate of entry of arsenite. *P* marks approximate age at pupation.

The data shown in Figure 1 were obtained with two lots of larvae. Incomplete runs with other lots indicated the same general variation with age, but the rate of entry of arsenic differed with the various lots. The range found for approximately 5-day-old larvae is discussed in the next section. It is probable that the same relation to age would be found at pH's other than 5, but no study was made of this point.

EFFECT OF PERIOD OF IMMERSION AND EXPOSURE TO SOLVENTS

If exposure of the larvae to the arsenite solutions for a period such as 24 hours resulted in a change in the permeability of the integument, it would not be proper to use average values for the total time concerned. Accordingly, tests were conducted with exposures of varying length. The results, given in Table 1, were obtained with groups of fifty larvae, analyzed five at a time, and are expressed in terms of arsenic entering a larva in 1 hour. This method will also be used later for data from tests in which, as in the present case, ratios only are important. Larvae of apparently uniform size were selected, but the tedious measuring was not carried out.

It is obvious that, at pH 5, exposure up to 25 hours caused no change in the rate of entry and that at pH 9.9 the change, if any, is very slight. However, at pH 11.5 a threefold increase is shown between exposures of $4\frac{1}{2}$ and 24 hours. It may be questioned whether the rate of entry remained constant even within the $4\frac{1}{2}$-hour period. On the basis of these results, rates obtained for periods of exposure up to 24 hours are accepted as correct with all pH's up to about 10.

The closely agreeing figures for rate of

entry of arsenic as given in Table 1 were obtained with three lots of larvae. Other lots were similarly independent of length of exposure at the lower pH's, but both lower and higher rates of entry were found. The ranges were 1.96–5.72 γ As/larva/hour at pH 5.0; 1.20–3.77 at pH 9.9; and 0.78–1.14 at pH 11.5 (with 24 hours' exposure). Thus, within a given lot, agreement was close, but variations occurred from one lot to another. To a small extent these were due to differences in size, but unknown factors, possibly related to the state of nutrition and rate of growth, appeared to be more important.

A further point of importance is the difference in the rates of entry of arsenic into individual larvae in a given test. Twenty larvae run at pH 5 and analyzed individually gave results varying from 2.2 to 7.6 γ As/larva/hour (mean 4.28). The range at pH 9.9 was from 0.7 to 2.8 (mean 1.42), and at pH 11.5 it was from 1.07 to 1.84 (mean 1.32). The standard deviations of the mean were 0.41, 0.24, and 0.14 γ As/larva/hour for pH 5, 9.9, and 11.5, respectively. These results show what may be regarded as the normal variation between individuals and indicate the necessity for using fairly large numbers in order to obtain a representative average result.

Since long immersion in alkaline solution of pH 11.5 increased the permeability of the integument to arsenic, it was of interest to study briefly a few typical fat solvents. A group of larvae was killed by exposure to chloroform vapor and, without ligation, was immersed in 0.223 M arsenite solution at pH 5 for 22½ hours. The average amount taken up was 3.08 γ As/larva/hour. Another group from the same lot of larvae was doubly ligated and found to take up 1.96 γ As/larva/hour. The average area per larva was slightly greater in the first case because the ends of the body were not tied off; but it is obvious that the chloroform had increased the rate of entry of arsenic by about 50 per cent.

Another experiment was made with groups of twenty-five to fifty larvae, in which the arsenic solution contained 2 per cent by volume of acetone or Cellosolve (ethyl ether of diethylene glycol) in one case and 50 per cent of the same materials in the other instance. As shown by the controls, the two lots of larvae used differed somewhat in their permeability to arsenic in the absence of the solvents. The data are given in Table 2.

TABLE 1

EFFECT OF PERIOD OF IMMERSION UPON RATE OF ENTRY OF ARSENIC INTO DOUBLY LIGATED LARVAE OF *S. securifera* VILL.

pH	Period of Immersion (Hours)	Average Rate of Penetration (γ As/Larva/Hour)
5.0	3	3.24
	6¼	3.48
	12	3.34
	14	3.26
	22¾	3.18
	25	3.20
9.9	4	1.43
	21	1.50
11.5	4½	0.26
	24	0.78

There is an indication of an increase in rate of entry during the 4-hour exposure to the 2 per cent acetone solution and an unquestionable increase in the case of the 27½-hour exposure to the 50 per cent solution. Cellosolve, on the other hand, had no significant effect for either period. The ranges in the rates of entry compare favorably with those found in other runs with doubly ligated larvae.

EFFECT OF ARSENIC CONCENTRATION

The possible effects of a variation in concentration of arsenite were investi-

gated by exposing doubly ligated larvae in groups of fifty for 24 hours to solutions at pH 5 containing 0.112 M, 0.128 M, and 0.223 M arsenite. The results, as shown in the third column of Table 3, indicate that, over this range and at the given pH, the amounts of arsenite entering the larvae are proportional to the concentration of the outside solution. No study was made at other pH values. This method of dividing the actual amount of arsenic entering per square millimeter per hour by the concentration gives the rate of entry from a hypothetical 1 M solution and is a very handy method of expressing results, as will be illustrated again later.

TABLE 2

EFFECT OF ACETONE AND OF CELLOSOLVE ON RATE OF ENTRY OF ARSENITE INTO LARVAE OF *S. securifera* VILL. EXPOSED AT pH 7

SOLVENT	CONCENTRATION (PER CENT BY VOLUME)	RATE OF ENTRY OF ARSENIC (γ As/Larva/Hour)		DURATION OF EXPOSURE (HOURS)
		Average	Range	
Acetone	2	3.67	2.8–4.3	4
Cellosolve	2	3.07	2.3–4.0	4
Water only		3.10	1.4–3.6	4
Acetone	50	3.56	2.0–4.5	27½
Cellosolve	50	2.60	1.6–3.6	27½
Water only		2.48	1.7–3.3	27½

TABLE 3

EFFECT OF VARIOUS CONCENTRATIONS OF ARSENITE AT pH 5 UPON RATE OF ENTRY INTO DOUBLY LIGATED LARVAE OF *S. securifera* VILL.

Concentration	Amounts Penetrating (γ As/Mm²/Hour)	Amounts Entering (γ As/Mm²/Hour/Concentration)
0.112 M.	0.00612	0.0546
0.128...	0.00720	0.0562
0.223...	0.0120	0.0540

PENETRATION THROUGH DETACHED INTEGUMENTS

Comparison of the amounts of arsenite penetrating doubly ligated larvae and detached integuments is slightly complicated by the circumstance that the arsenic remaining in the integument was included in the case of the former but not of the latter. In order to determine the correction for arsenic retained, a group of thirty-five doubly ligated larvae in lots of five was exposed to 0.233 M arsenite at pH 5 for 21¾ hours, removed, washed, the ends cut off, and the body contents removed as in preparing isolated integuments. Separate weighing and analysis of the integuments, of the other body solids, and of the body fluids gave the following average weights and contents of arsenic: integument 0.0197 gm., 23.3 γ As; other body solids 0.0837 gm., 76.2 γ As; body fluids 0.0556 gm., 45.4 γ As. Hence it may be calculated that 16.1 per cent of the total arsenic was retained in the integument. It should be noted that this figure can be valid only for the particular treatment used in preparing the isolated membranes, and an integument from which the jelly-like layer had been removed doubtless would retain less arsenic. Also the time of immersion in the

arsenic solution and the pH of the latter may be of importance. The average weights given are for the condition at the end of exposure, and probably the ratios would be changed with larvae which had been in drier surroundings. The weight of body fluids was determined by difference, since evaporation from the filter paper on which they were absorbed would be very difficult to prevent.

Similar tests with larvae immersed for $21\frac{3}{4}$ hours in arsenite solution at pH 9.9 indicated that 11.4 per cent of the total arsenic taken up was retained in the integument. No comprehensive study was made to determine whether there is a real difference between the behaviors at pH 5 and at pH 9.9, but the correction in either case is relatively small.

The amount of arsenite penetrating *through* the integument of doubly ligated larvae may be found by subtracting that retained in the integument from the total taken up. Comparative results with doubly ligated larvae and isolated integuments are shown in Table 4. It seems certain that there is no real difference in the amounts of arsenic penetrating through the integument in the two cases. In all later work the total taken up was used. In a number of instances the membranes exposed at pH 11.5 underwent a change in appearance, and some were found to be broken. This may be compared with the behavior of larvae exposed at the same pH.

EXPERIMENTS WITH SINGLY LIGATED LARVAE

A series of tests was made with larvae ligated at the head end only, excretion thus being allowed during exposure to the arsenite solution. Four runs of 4 hours each, involving a total of one hundred and six larvae from four different lots, resulted in accumulation at the rates of 1.64, 1.87, 2.47, and 3.03 γ As/larva/hour at pH 5.0. Similarly, two runs at pH 9.9 gave 0.78 and 1.05 γ As/larva/hour. Assuming that rate of entry of arsenic was not affected by the method of ligation, comparison of these figures with those for the tests with doubly ligated larvae at the same pH values (Table 1) indicates that considerable excretion of arsenic occurred during the 4-hour exposure. If larvae from the same lots are compared, it appears that about 40 per cent of the total arsenic taken in was excreted during the period of exposure at each pH.

TABLE 4

COMPARATIVE RATES OF PASSAGE OF ARSENITE THROUGH INTEGUMENTS OF DOUBLY LIGATED LARVAE AND THROUGH DETACHED INTEGUMENTS

pH	Period of Exposure (Hours)	Arsenic Penetrating in—	
		γ As/Integument/Hour	γ As/Larva/Hour
5.0	26	2.21	2.12*
	$21\frac{3}{4}$	5.6	5.5 †
9.9	$21\frac{3}{4}$	1.12	1.10‡
11.5	23	0.88	0.83§

* Using correction factor of 16.1 per cent held in the integument.

† These runs were made with younger larvae.

‡ Using correction factor of 11.4 per cent held in the integument.

§ No correction factor was determined for this pH, but that from pH 9.9 was assumed.

In some runs the singly ligated larvae were taken from the arsenite solution, washed, and placed in dry sand after removal of the ligature. Within a few days the survivors would pupate. Analyses were made of the arsenic remaining in the pupae and in the dead larvae. Typical data are shown in Table 5.

A total of twenty-seven pupae was analyzed, most of them individually, and the highest amount of arsenic found in any one was 3 γ in a pupa of abnormal appearance. These results indicate that only those larvae which excreted almost all the arsenic taken in during immersion were able to pupate. A possible alternative explanation is that the pupae came

from larvae which took in but little arsenic. Individual analyses were made of a group of singly ligated larvae exposed to 0.223 M arsenite at pH 5.0 for 4 hours and of another group exposed at pH 9.9. The amounts of arsenic found varied from 7.8 to 60.8 and from 3.2 to 15.0 γ, respectively. The variation is larger than with doubly ligated larvae (cf. p. 263), but the very lowest values are much greater than those for the pupae from larvae surviving similar exposures, as shown in Table 5. Hence excretion doubtless occurred following removal from the arsenite solution after exposure for only

TABLE 5

AMOUNTS OF ARSENIC FOUND IN DEAD LARVAE AND IN PUPAE FROM LARVAE SURVIVING EXPOSURE TO ARSENITE SOLUTIONS WHILE SINGLY LIGATED

pH	γ As/Larva	γ As/Pupa
5.0.....	12.1	1.0
9.9.....	10.2	0.7
11.5.....	10.0	0.2±

4 hours. A more complete analysis was made with one group of thirty-two larvae exposed at pH 11.5 for 4 hours. Two pupae resulted, and they contained 0.2 $\pm$ γ As each. The thirty dead larvae contained 241 γ As, or 8.0 γ each. The surrounding sand contained 104 γ As, which had been excreted by the two larvae before pupation and by the others before death. Assuming that the pupae had originally contained the same amount as the others and subtracting the additional arsenic excreted by them, i.e., 2 $\times$ (8.0–0.2) or 15.6 γ, from the total excreted, there remains 88 γ As from thirty-two larvae, or an average excretion of 2.8 γ. The original content, then, would be 10.7 γ, and about 26 per cent was excreted, on the average, before death after removal from the arsenite solution. This is in addition, of course, to that excreted during exposure. If the pupae came from the larvae which took up the least arsenic, this would change the calculations to indicate a slightly greater excretion by the larvae before death.

EFFECT OF pH UPON RATE OF PENETRATION

In a number of cases, larvae of the same lot were doubly ligated and run at more than one pH. It was of interest to compare the corresponding amounts of arsenic found at the end of the period of immersion. In particular, it was possible to determine how closely the change in rate of entry of arsenic agreed with the change in concentration of molecular arsenious acid. Since the various lots of larvae exhibited considerable variation in rate of entry, it was impracticable to show the effect of alteration in pH by direct use of the data as they have been expressed previously. Instead, the rate of entry for each lot at pH 5 was taken as 100 per cent, and the rates at other pH's were expressed in their corresponding percentages. As an example, the data for one lot of larvae were: at pH 5.0 the rate of entry of arsenite was 2.5 γ As/larva/hour and at pH 10 it was 0.63 γ As/larva/hour. These are in the ratio of 100:25.2 per cent. The data expressed in this manner are shown in Figure 2, and curve *A*, drawn by eye, shows the trend of the results. Figure 2 also includes a titration curve, *B*, for arsenious acid. From the relation $pH = pK_a + \log(\text{salt/acid})$ it is possible to calculate the fractions present as arsenious acid and as arsenite ion. There is some uncertainty regarding pK_a for arsenious acid, the values given by different experimenters varying from 2.1×10^{-8} to 2.4×10^{-10}. The latter figure is the most recently determined

(Garrett *et al.*, 1940) and was chosen as probably the most accurate. Variation in the value used for pK_a does not affect the shape of the titration curve but only shifts it slightly to the right or left as higher or lower values are used. The presence of polymers, e.g., $H_2As_2O_4$ and $H_3As_3O_6$, as found to occur in arsenite solutions by Garrett *et al.*, does not appreciably affect the shape of curve *B*, since it may be calculated from the data

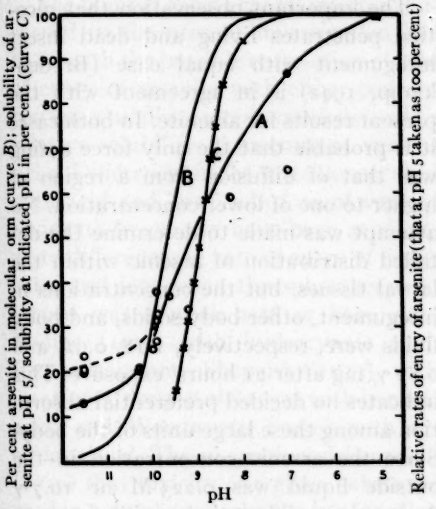
Per cent arsenite in molecular form (curve B); solubility of arsenite at pH 5/ solubility at indicated pH in per cent (curve C)
Relative rates of entry of arsenite (that at pH 5 taken as 100 per cent)
pH
A
B
C

FIG. 2.—Relation of relative rates of penetration of arsenite into fly larvae (*A*) to degree of ionization (*B*) and relative solubilities (*C*).

of Garrett *et al.* that these acids have nearly the same dissociation constant as has ordinary arsenious acid. It may be noted that, in the range of pH from 5 to 11.5, arsenite changes from undissociated acid to almost wholly ionized salt.

Figure 2 also contains a curve, *C*, showing the relative solubility of arsenious acid at various pH values. This is included in order to examine the suggestion of Burtt (1945) that toxicity and perhaps penetration may be related to relative solubility of the toxicant in the solution used in the exposure. The necessary data may be found in Table II as given by Garrett *et al.* (1940) in terms of mols of As_2O_3 soluble in 1,000 gm. of water containing given amounts of sodium hydroxide. By plotting the values given and interpolating at intermediate concentrations of sodium hydroxide, the first and second columns of Table 6 were obtained. The corresponding pH values (third col.) were calculated, with the assumption that the ionic strengths of the various solutions were

TABLE 6

SOLUBILITY OF ARSENIOUS ACID AT VARYING pH VALUES (INTERPOLATED FROM DATA OF GARRETT *et al.*, 1940)

Sodium Hydroxide (Mols/1,000 Gm Water)	Arsenious Acid (Mols/1,000 Gm Water)	pH	Solubility of Arsenite at pH 5 ÷ Solubility of Arsenite at Given pH (Per Cent)
0	0.207	5.11*	100
0.01	0.222	7.95	93.7
0.03	0.247	8.39	84.3
0.05	0.277	8.59	74.9
0.07	0.308	8.71	67.5
0.1003	0.3504	8.84	58.4
0.15	0.436	8.99	47.7
0.20	0.520	9.10	40.1
0.3	0.670	9.23	31.2
0.4	0.826	9.32	25.2
0.6	1.140	9.43	18.3

* Calculated from solubility, 0.208 mols/1,000 gm water, and dissociation constant, 2.4×10^{-10}. This same value for solubility holds at pH 5.0 according to the data of Garrett *et al.* (1940).

due entirely to the sodium arsenites and that the polymerized forms of the latter, as found by Garrett *et al.*, exist as monovalent ions. Hence the ionic strengths are numerically equal to the concentrations of added sodium hydroxide.[2] In the penetration experiments the buffer salts also were present, but their effect on the ionic strength is slight, and, as a matter of fact, all solutions were dilute enough

[2] As a sample calculation, take the case in which added sodium hydroxide equals 0.20 M and the experimental (OH^-) is 2.1×10^{-5} M. Ionic strength, μ, equals 0.20; $\log \gamma_{OH^-} = -0.505 \times \sqrt{\mu} = -0.226$. Hence $\gamma_{OH^-} = 0.594$ and $a_{OH^-} = 2.1 \times 10^{-5} \times 0.594 = 1.25 \times 10^{-5}$, and $\log a_{OH^-} = \bar{5}.10$. Hence $pH = -\log a_{OH^-} = 14 + \log a_{OH^-} = 9.10$.

so that hydroxide ion concentrations as given by Garrett *et al.* could have been used instead of activities, with an error of only a few tenths of a pH unit.

The ratio of solubility of arsenite at pH 5 to solubility at other pH's is calculated from the data of Garrett *et al.*, that at pH 5 being taken as 100 per cent. Since the solutions at pH 5 all contained more arsenious acid than Garrett *et al.* (1940) found to dissolve in pure water, it is probable that the 0.2 M phosphate buffer increased the solubility enough to cover the slight increase. A similar effect may be expected over the pH range used; hence the relative solubilities probably are not seriously affected. Any error introduced by the fact that Garrett *et al.* based concentrations on 1,000 gm. of water, whereas in the present work they were based on a 1,000-cc. solution, is entirely negligible at the low concentrations used.

DISCUSSION

The relatively high permeability of the integument of young Sarcophagid fly larvae in comparison with later stages as revealed by the present experiments is similar to the finding of Lennox (1940) with larvae of *Lucilia cuprina* exposed to soluble arsenic and of Morozov (1935), who exposed larvae of *Loxostege sticticalis* and *Pieris rapae* to acetic acid and ammonia. Reduction in the permeability of the integument as the larva grows older is probably a general rule and is in line with the well-known fact that contact insecticides are more effective against young larvae.

In order to express the rate of penetration of substances through insect cuticle in units which allow comparison with other organisms (cf. Brooks and Brooks, 1941, chaps. ix and x), it is proposed that the rate be stated as micrograms/mm²/hour/concentration. In the case of larvae of *S. securifera*, this quantity decreases from 0.31 for 36-hour larvae to 0.055–0.065 for 100–180-hour larvae and rises to about 0.15 for pupae a day or two old, exposure being made to arsenite solutions held at pH 5.0. Average values at other pH's for fully fed larvae, calculated from curve *A* of Figure 2, are: pH 7, 0.051; pH 9, 0.028; pH 10, 0.015; pH 11.5, short exposure 0.009, long exposure 0.012.

The important observation that nicotine penetrates living and dead insect integument with equal ease (Bredenkamp, 1942) is in agreement with the present results for arsenite. In both cases it is probable that the only force acting was that of diffusion from a region of higher to one of lower concentration. No attempt was made to determine the detailed distribution of arsenic within the larval tissues, but the concentrations in integument, other body solids, and body fluids were, respectively, 1.18, 0.91, and 0.82 γ/mg after 21 hours' exposure. This indicates no decided preferential absorption among these large units of the body. Since the arsenic concentration in the outside liquid was 0.223 M or 16.7 γ As/mg, it is evident that equilibrium was far from being reached within the relatively long period of exposure. Hence any change in the ratio of molecules to ions that might occur after penetration, as suggested by Brooks and Brooks (1941, p. 149), will have negligible effect upon the accumulation of arsenic within the larvae. The comparatively low concentration reached within the body doubtless accounts for the fact that no diminution in the rate of entry was found for at least 24 hours. When excretion was permitted and the larvae survived, all tissues became almost completely free from arsenic. Hence only a minute amount, at most, could have been in an insoluble or nonreleasable combination. Elimination

of absorbed arsenic undoubtedly is the determining factor for survival when amounts not too greatly exceeding the median lethal dose are concerned. A similar balance between incoming and outgoing arsenic has been reported by Voskresenskaya (1936) for cutworms fed sodium arsenite and by O'Kane and Glover (1936), who applied soluble arsenites to the integument of American roaches.

The point of most general interest concerned in the present investigation has to do with the possible correlation of rate of entry of arsenite with either concentration of undissociated arsenious acid or relative solubility of arsenite at various pH's. Differences in the toxicities secured with acid and basic solutions of various toxicants frequently have been attributed to differences in rate of entry of molecules and ions when the substance concerned was a weak acid or base, e.g., Bodine (1924) with hydrocyanic acid, Richardson and Shepard (1930) with nicotine, Cruess *et al.* (1931) with benzoic acid, and Woodworth (1938) with arsenious acid. The suggestion of Ferguson (1939) that toxicity of a given substance used in a number of solvents is a function of its chemical activity and hence should vary inversely to its solubility in the solvents was used by Burtt (1945) in explaining the relative toxicities to ticks of phenol in various media. He extended the idea to include the variation in toxicity of aqueous arsenite solutions at different pH values and suggested that rate of penetration through the integument might be a function of the solubility at various pH's.

It will be recalled that Figure 2 was constructed by the method of taking the rate of entry of arsenic through fly-larval integument as 100 at pH 5 and similarly taking the solubility as 100 at this same pH. Without any assumptions, the percentage of arsenic in the form of nonionized arsenious acid is essentially 100 at this point. The three curves for these respective functions show that there is numerical agreement at pH 5 (by convention) and between the titration and penetration curves in the region of pH 9.5–10 but that the relative rate of entry is low over the range between pH 5 and pH 9.5 and high in strongly alkaline solutions. The relative solubility and the penetration curves show less similarity. It may be noted that the general shape of curves *A* and *C* is not dependent upon this method of expression but is the same if actual quantities are plotted. No known property of arsenite solutions seems capable of accounting quantitatively for this relation between the functions, though the high penetration in the alkaline region may be accounted for partially by the calculations of Garrett *et al.* (1940), which indicate considerable polymerization to $HAs_2O_4^-$ and $H_2As_3O_6^-$ or their acids at the concentration used. While these compounds contain two and three times as much arsenic as ordinary arsenite, they probably penetrate somewhat less readily.

Alteration in the intrinsic permeability of the integument at the various pH's seems to offer a more probable explanation of the experimental data than does any property of arsenic. Thus it was mentioned earlier that strongly alkaline solutions visibly injured the integument after prolonged exposure, and it was found that the average rate of entry of arsenic at pH 11.5 was three times as great during a 24-hour exposure as during one of 4 hours. It is possible that invisible changes occurred during the shorter period which may account for the departure of curve *A* from *B* and *C* at high pH values. However, this hypothetical alteration would have to be greater than that occurring subsequently during

the 4–24-hour interval. The importance of the lipids present in the integument is indicated by the observation that chloroform or acetone are able to increase the entry of arsenic to a considerable degree. Similar observations have been made by many experimenters, e.g., the loss of water from various insects has been correlated with the nature of the waxy layer comprising the outer part of the integument (Wigglesworth, 1946).

The lowered rate of penetration in the neighborhood of neutrality does not seem to be attributable to any known property of the integument. Yonge (1936), in a study of the permeability of lobster chitin to several molecular and ionic substances, found evidence for an isoelectric point near pH 3.5, with low penetration in this neighborhood. It is unlikely that insect chitin has an isoelectric point near neutrality, as would be necessary to explain the penetration curve, nor is alteration of the lipid layer to be expected near neutrality. The question may be raised as to whether the third well-recognized constituent of insect cuticle—protein—may exert a special effect in the pH range concerned. The work of Fraenkel and Rudall (1940) resulted in their hypothesis of the cuticle below the lipid layer as dispersed chitin in a continuous phase of hydrated protein. The passage of water-soluble arsenious acid through such a system will depend largely upon the degree of hydration of the protein, which, in turn, varies with the pH. If the protein concerned has an isoelectric point near neutrality, it will have a minimum degree of hydration in this neighborhood and hence be least permeable.

The experimental fact that over a wide range of pH the same rate of penetration of arsenic has been found with doubly ligated larvae, which had a relatively constant pH near neutrality at the interior of the cuticle, and with detached integuments, which were exposed to the various pH's on both sides, is an indication that the region affected by pH changes is not near the inner surface. It was often observed that after exposure to alkaline solutions the integument separated into two layers, in addition to the jelly-like one. While no measurements of thickness were made, the outer one obviously was much thicker than the lipid layer described by many authors.

Trim (1941) has reported analyses for a protein fraction from Sarcophagid larval cuticle readily soluble in water and for another which dissolves after prolonged treatment with warm 5 per cent sodium hydroxide solution. The latter fraction is intimately associated with chitin. The degree of hydration, swelling, and permeability of these protein constituents apparently have not been studied over a range of pH, and at present it is possible only to suggest that they may have an effect upon the entrance of such substances as arsenites. In particular, the protein in the layer nearest the outer surface of the cuticle seems worthy of study from this point of view.

The foregoing discussion has ignored the possibility that an appreciable amount of arsenic penetrates larval cuticle in the form of arsenite ions. In particular, it may be argued that a part, at least, of the relatively great penetration in the strongly alkaline pH range is due to this form. The quantitative correlation of rate of entry with concentration of molecular arsenious acid in the region of pH 9.5–10, at which about 70 per cent of the total arsenic is in ionized form, is conclusive proof that ionic penetration cannot be extensive at this pH or in the lower range. The quantitative agreement between rate of entry of arsenic and concentration of molecular arsenious acid goes beyond most results reported previously (e.g., Richardson and Shepard,

1930; Hoskins, 1932; Woodworth, 1938), since these showed only a general qualitative agreement between the two factors.

The increased rate of penetration found with pupae is surprising in the light of Fraenkel and Rudall's observation that the integument loses water as pupation occurs. Barring other changes, this would be expected to reduce the entry of arsenic in aqueous solution. Immersion of the pupae in the aqueous solutions doubtless resulted in considerable swelling, although, according to Fraenkel and Rudall (1947), this is mostly perpendicular to the surface and not likely to increase permeability. The experiments involved 45 pupae of each age, and the results were more consistent than were those usually obtained with larvae, e.g., the range for the newly formed pupae was 0.014–0.0512 γ As/mm^2/hour and for those 2 days older 0.0269–0.0337 γ As/mm^2/hour. On the basis of these limited experiments it may be suggested that there occur other changes than those mentioned by Fraenkel and Rudall—for instance, the lipid layer may be altered.

The present work shows that arsenite is not a suitable toxicant with which to choose between the molecular-concentration and the relative-solubility theories of penetration as outlined by Burtt (1945). The two properties vary in a similar manner with pH and either will serve as an explanation of the results on rate of penetration in conjunction with some assumptions concerning changes in the cuticle, as discussed before.

SUMMARY

Larvae of the large fly, *S. securifera*, have been used in a study of the rate of penetration of trivalent arsenic in aqueous solution through insect integument over a pH range of 5.0–11.5, within which the solute changes from undissociated arsenious acid to highly dissociated sodium arsenite. The rate of penetration is highest in young larvae, decreasing to a steady value with larvae 100–180 hours old and rising somewhat at pupation. The rate was not appreciably affected by exposure up to 24 hours, except at the highest pH, and was directly proportional to the concentration. Fat solvents either applied first or included in the arsenic solution increased the rate of its penetration. No detectable difference was found over the given pH range between larvae ligated at both ends and detached integuments. Hence no "living" effect occurred. If larvae were ligated only at the head end, excretion occurred during exposure and continued until an extremely small amount of arsenic was left in pupae from surviving larvae.

In order to study the correlation of rate of penetration with concentration of molecular arsenious acid, both were taken as 100 per cent at pH 5.0. A plot of these two quantities versus pH gave the ordinary sigmoid titration curve; and a rate-of-penetration curve, which was relatively low over the neutral range, was in agreement with the titration curve at about pH 10 and was relatively high in stronger alkali. It is concluded that rate of penetration would be proportional to concentration of nonionized arsenious acid, except for alteration of the lipid layer of the integument in the presence of fairly strong alkali and some unknown change in the cuticle, probably in the protein constituents, occurring near neutrality. Rate of penetration is less well correlated with relative solubility over the pH range concerned, but arsenite does not appear to be a good toxicant for use in choosing between the two hypotheses.

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THE SEASONAL REPRODUCTIVE CONDITION OF MALE BROWN RATS IN BALTIMORE, MARYLAND[1]

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A STUDY of the reproduction of Brown Rats (*Rattus norvegicus*) throughout the year is of value because this species, in contrast to most rodents, may reproduce in all months. This paper describes the condition of the reproductive organs of male rats captured from comparable habitat conditions during a period of 14 months. A secondary objective is to develop some simple field criteria of reproductive activity or condition. Another paper will describe the reproduction of the females.

It might be expected that the testes of male feral Brown Rats would ascend into the abdomen in the winter and become aspermatic and, further, that the secondary sex organs would consequently regress, since such seasonal regression is

[1] Contribution from the Rodent Ecology Project of the Department of Parasitology, School of Hygiene and Public Health, The Johns Hopkins University. The work reported in this paper was conducted under a grant from the International Health Division of the Rockefeller Foundation.

common in rodents such as *Microtus* (Hamilton, 1941; Rowlands, 1936). It was noticed, however, that feral male Brown Rats living out of doors in the city of Baltimore and on a farm near by had active testes throughout the winter, in contrast to field mice (*Microtus*) trapped near by, which had aspermatic testes. An investigation of the condition of the reproductive organs of Brown Rats was conducted to determine the activity of the testes and the condition of the secondary sex organs in every month of the year.

The extensive literature about seasonal reproduction of wild rats, as summarized by Buxton (1936), was based almost entirely on females, but Perry (1945) studied the reproductive organs of male Brown Rats in England and found no seasonal variation. Davis (1947, 1948) found no evidence of seasonal changes in reproductive organs of male Roof Rats (*Rattus rattus*) on farms in Texas and of male Brown Rats and Roof Rats in San Antonio, Texas, and concluded that any seasonal changes in pregnancy rates were dependent upon the females. A serious deficiency of most studies was that the rats came from various habitats at different seasons of the year and were thus hardly comparable.

MATERIALS AND METHODS

The rats for these studies were obtained in wholesale poultry warehouses in Baltimore, Maryland. These warehouses were selected because they provided a homogeneous habitat with the largest population of rats. The few grain warehouses had rats, and one such warehouse was used because its grain and other ecological conditions were similar to those of poultry warehouses. The poultry warehouses are located in old buildings with ample harborage for rats in the walls and under piles of junk. There is plenty of water and an abundant supply of food in the form of poultry feed and an occasional dead bird.

The rats were captured at regular intervals of 3–5 weeks in each warehouse in rotation. After trapping had been carried on in one warehouse for a week or two, the traps were removed and set in other warehouses until it was time to return to the first warehouse. By this method rats were obtained in every week of the year, and yet the population of rats was not depleted in any warehouse. Fifteen poultry warehouses were used.

The traps were No. 0 steel traps, set unbaited in runways. The rats were removed alive from the traps and brought to the laboratory for autopsy. A total of 721 male rats was autopsied.

The autopsy data were coded and put onto cards for mechanical sorting and tabulation. For the males the following data were obtained: location of capture; date; weight; body plus head length; tail length; third molar (erupted or not); skull (ossified or not); testes length; testes position (abdominal or scrotal); character of spermatic artery (absent, single, or branched); testes color (gray, pearl, or black); weight of Cowper's gland; seminal vesicles length; seminal vesicles shape (straight, convoluted, or balloon); ventral prostate size; seminiferous tubules (visible or not). The testes, ventral prostate, Cowper's gland, and seminal vesicles were fixed in Bouin's solution.

After fixation, the testes, seminal vesicles, and Cowper's glands were cleaned of excess tissue and weighed to the nearest milligram. The weights in this paper refer to one testis, seminal vesicle, or Cowper's gland, not to the pair. A part of each testis was sectioned at 9 μ and stained in haematoxylin and eosin. The spermatozoa were then classified as absent, few, or abundant.

SEASONS OF THE YEAR

In order to divide the year into biologically useful parts, a hythergraph (Fig. 1) was constructed to show the weekly mean temperatures and total weekly precipitation from January 1, 1946, to March 3, 1947. From this graph it was possible to divide the year into seven seasons, each having relatively homogeneous temperature and rainfall conditions. Although such biological seasons cannot adequately describe the impact of weather upon the physiology of an animal, these seasons are better than the conventional astronomical seasons.

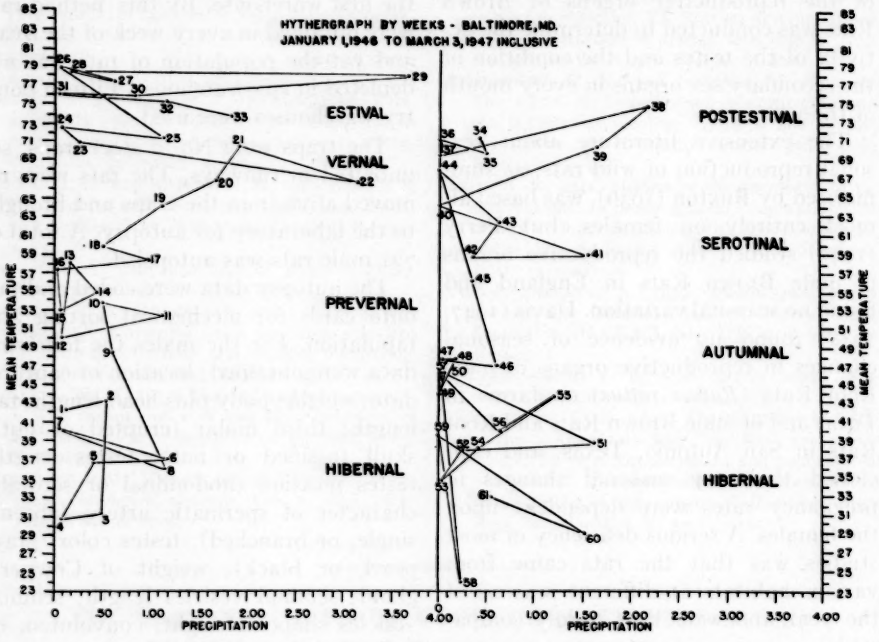

FIG. 1.—Hythergraph of the weekly temperature and precipitation at Baltimore, January 1, 1946–March 3, 1947.

The temperature (degrees F.) and precipitation (in inches) records are summarized in Table 1. The seasons may be briefly characterized as follows: The *hibernal season* (1946), when the collections began, was cold and had considerable precipitation; the *prevernal season* was warmer and had about the same precipitation; the *vernal season* was still warmer and had heavy rainfall in several weeks; the *estival season* was hot, with considerable rain; the *postestival season* was slightly cooler, with several very dry weeks; the *serotinal season* was cooler and moderately wet; the *autumnal season* was short, cool, and dry; the *hibernal season* (1946–47) was cold and wet.

The weather conditions in the poultry warehouses reflect external conditions, since the doors are usually open and there are no central heating units. The temperature at all times is less variable than is the external temperature, and in winter the buildings are always kept

above freezing by wood-burning stoves. Because of washing floors and scalding poultry, there is a high humidity. In summary it can be said that the weather within the poultry warehouses changes from season to season, is less extreme than external weather, but is much more variable than weather in a restaurant or hotel.

COMPOSITION OF THE MATERIAL

The rats used in this investigation are indicated by weights and seasons in Table 2. The number of rats captured differs from season to season, and the percentages in the various weight classes show great variability (Fig. 2). The high percentages of small rats in the prevernal and vernal seasons and then again in the postestival and serotinal seasons suggest an increase in reproduction at these times. But the high percentage of young rats in the hibernal 1946 season is unexplained and is in striking contrast to the low percentage in the hibernal 1947 season.

TABLE 1

MEAN TEMPERATURE AND TOTAL PRECIPITATION FOR BIOTIC SEASONS

Season	Dates	Weeks	Mean Temp. (° F.)	Precipitation (Inches)
Hibernal (1946)..	Jan. 1–Feb. 25, 1946	1–8	37.2	4.23
Prevernal.....	Feb. 26–May 6	9–19	54.5	4.52
Vernal.....	May 7–June 24	20–25	68.9	9.44
Estival.....	June 25–Aug. 19	26–33	76.7	8.59
Postestival.....	Aug. 20–Sept. 30	34–39	71.2	4.85
Serotinal.....	Oct. 1–Nov. 11	40–45	61.4	2.95
Autumnal.....	Nov. 12–Dec. 16	46–50	46.6	0.94
Hibernal (1947)..	Dec. 17–Mar. 3, 1947	51–61	37.3	6.66

SEASONAL CONDITION OF THE TESTES AND SECONDARY SEX ORGANS

The analysis of seasonal reproductive condition in male rats requires consideration of testicular activity from season to season both directly in terms of size and sperm production and indirectly in terms of the secondary sex organs. Therefore, this section describes the condition of the testes, seminal vesicles, ventral prostate, and Cowper's glands throughout the year.

1. *Testes.*—Table 3 shows the relation between weight of one testis and weight class of rat for the various seasons. This table is compiled from tables giving an even more detailed division of the weight classes. It is at once apparent that there is no seasonal change in the weight of the testes, for in every season large rats have large testes and small rats have small testes. If there were a seasonal change, then large rats in some season (hibernal, probably) would have small testes. Similar tables for the relation between the length of the testis and the weight of the rat, for the relation between the weight of the testis and the length of the rat, and for the relation between the length of the testis and length of the rat, all show that there is no change in the size of the testes according to seasons.

The relation between body weight of rats and the presence of sperm is shown in Table 4, which is a summary of a table showing the data by seasons. It is again at once apparent that there is no seasonal change in the presence of sperm, because large rats collected in all months of the year had sperm. A similar table showing the relation between the presence of

sperm and body length confirms this conclusion.

The relation between the position of the testes (abdominal or scrotal) and the weight of the rat is shown in Table 5. the inguinal canal. If the testes were in the abdomen but could be pushed through the inguinal canal into the scrotum, then the testes were recorded as scrotal. Table 5 indicates an abdominal

TABLE 2

COMPOSITION OF SAMPLE BY WEIGHTS AND SEASONS

Season	Male Rats	Percentage in Weight Class (Gm.)					
		0–99	100–199	200–299	300–399	400–499	500–599
Hibernal (1946)	117	49.5	13.7	12.8	14.5	8.5	1.0
Prevernal	152	32.2	20.4	12.5	22.4	11.2	1.3
Vernal	76	50.0	14.5	17.1	13.2	5.2	0
Estival	101	13.9	26.8	20.8	31.6	6.9	0
Postestival	74	28.4	18.9	12.2	17.5	20.1	2.9
Serotinal	102	33.3	26.5	17.7	16.7	4.9	0.9
Autumnal	44	18.2	27.3	20.2	20.2	11.4	2.7
Hibernal (1947)	55	7.3	16.4	18.1	32.7	26.5	0

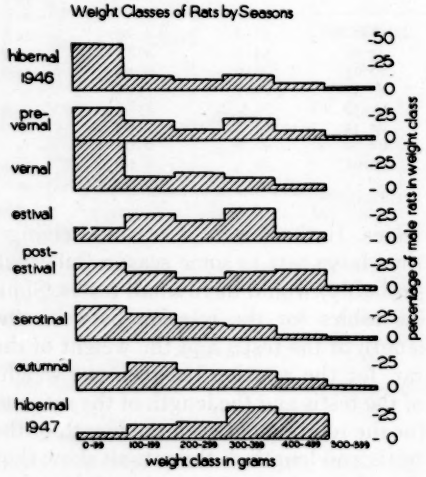

FIG. 2.—Percentage distribution of weight classes of rats by biotic seasons.

Again it is clear that there can be no retraction of the testes in any season, since large rats nearly always have scrotal testes. A similar table for the relation of testes position to body length supports these conclusions.

The standard used to determine the position of the testes was the condition of

condition for a few large rats (300–499 gm.). It seems likely that these testes were really scrotal but that an error was made because rigor mortis or some condition due to death caused an apparent closure of the inguinal canal. This technical error may occur in the whole series but would affect only a few individuals in each group. Thus, in the rats weighing 200–299 gm., about 40 per cent had truly abdominal testes which could not be pressed through the inguinal canal. It is not surprising that rats in the same weight class differ in respect to position of testes because the growth rate of wild rats is extremely variable (Davis and Winsor, 1948).

2. *Seminal vesicles.*—The relation between the weight of the seminal vesicles and the weight of the rat is shown in Table 6. It is obvious that there is no seasonal variation in this secondary sex organ, since large rats, collected in all seasons of the year, always had large seminal vesicles. The presence of sperm (Table 6) confirms this conclusion.

Other tables show that the shape of the seminal vesicles (straight, convolut-

TABLE 3

WEIGHT OF TESTIS ACCORDING TO SEASONS

Season	No. of Rats	Body-Weight Class (Gm.)	Percentage of Rats in Testis Weight Class (Gm.)		
			0.00–0.99	1.00–1.99	2.00–2.99
Hibernal (1946)	55	0–199	98	2	0
	32	200–399	19	81	0
	11	400–599	9	91	0
Prevernal	76	0–199	97	3	0
	53	200–399	10	90	0
	18	400–599	0	100	0
Vernal	45	0–199	96	4	0
	23	200–399	13	87	0
	4	400–599	0	100	0
Estival	40	0–199	98	2	0
	53	200–399	10	88	2
	7	400–599	0	100	0
Postestival	33	0–199	97	3	0
	21	200–399	0	100	0
	17	400–599	0	88	12
Autumnal	61	0–199	97	3	0
	34	200–399	15	75	10
	6	400–599	0	84	16
Serotinal	17	0–199	88	6	6
	18	200–399	22	78	0
	5	400–599	0	100	0
Hibernal (1947)	13	0–199	100	0	0
	27	200–399	11	85	4
	14	400–599	0	79	21

TABLE 4

RELATION BETWEEN BODY WEIGHT AND PRESENCE OF SPERMATOZOA

Presence of Spermatozoa	Body-Weight Class (Gm.)						
	0–99	100–199	200–299	300–399	400–499	500–599	Totals
None	193	85	1	2*	0	0	271
Few	1	28	8	2	0	0	39
Many	3	27	103	144	75	7	359

* Both these testes were pathologic on histologic examination.

TABLE 5

RELATION BETWEEN BODY WEIGHT AND POSITION OF TESTIS, ERUPTION OF THIRD MOLAR, AND OSSIFICATION OF SKULL

Body Weight (Gm.)	Testis Position		Third Molar		Ossification	
	Rats	Per Cent Scrotal	Rats	Per Cent Erupted	Rats	Per Cent Ossified
10–19	1	0	1	0	1	0
20–29	5	20	5	20	5	0
30–39	19	21	20	15	19	10
40–49	49	20	50	6	53	2
50–59	53	46	39	15	53	2
60–69	28	43	16	56	31	10
70–79	21	29	19	74	22	9
80–89	27	37	21	81	29	34
90–99	10	50	11	100	11	55
100–109	22	71	21	100	23	78
110–119	15	40	14	100	14	100
120–129	16	56	18	100	18	84
130–139	7	86	7	100	7	100
140–149	18	50	18	94	19	89
150–159	15	40	15	100	15	86
160–169	15	80	16	94	16	88
170–179	13	86	15	100	15	94
180–189	9	66	10	100	10	90
190–199	6	84	7	100	7	84
200–299	105	60	114	100	114	99
300–399	140	93	149	100	149	99
400–499	75	93	76	100	77	99
500–599	6	100	7	100	7	100

TABLE 6

RELATION OF SEMINAL VESICLE WEIGHT TO BODY WEIGHT AND SPERMATOZOA

Weight of Seminal Vesicles (Gm.)	Weight Class of Rat (Gm.)						Spermatozoa		
	0–99	100–199	200–299	300–399	400–499	500–599	None	Few	Many
0.00–0.09	195	135	32				274	35	48
0.10–0.19		5	13					1	16
0.20–0.29			9	5	1				15
0.30–0.39			12	10	2				24
0.40–0.49			10	16	5	1			32
0.50–0.59			9	35	3			1	46
0.60–0.69	1	1	8	19	17				46
0.70–0.79			4	27	7	2	1		38
0.80–0.89			6	16	15	1	1		37
0.90–0.99			5	12	11	2		1	29
1.00–1.09			1	5	5	1			13
1.10–1.19			2	2	3			1	6
1.20–1.29				1	2				3
1.30–1.39					2				2
1.40–1.49									
1.50–1.59			1						1
1.60–1.69					1				1
1.70–1.79									
1.80–1.89									
1.90–1.99					1				1

ed, or balloon) is worthless as a measure of sexual activity because shape shows little correlation with weight or presence of sperm.

3. *Ventral prostate.*—The relation between the size of the ventral prostate and the weight of the rat (Table 7) shows that there is no seasonal change in the prostate, for large rats have large prostates and the presence of sperm is correlated with prostate size.

4. *Cowper's gland.*—The relation between the weight of the Cowper's gland and the weight of the rat (Table 8) shows no seasonal change in this secondary sex organ, since large rats, caught in all seasons of the year, have large Cowper's glands. Furthermore, the presence of sperm is correlated with the size of this gland.

CRITERIA FOR ACTIVITY OF TESTES

A secondary object of this study is to develop simple criteria for activity of testes which can be used in field work. The length of the testes (Table 9) is correlated with the presence of sperm, but there is a considerable spread and overlap. A similar table for the relation of testis weight and the presence of sperm also shows considerable overlap. Furthermore, since these two criteria require a certain amount of technical labor, they are not recommended for field use.

The visibility of tubules (Table 9) is a satisfactory criterion of the presence of sperm, as Hamilton (1941) found in *Microtus*. The tubules when present are clearly visible to the naked eye through the shiny covering of the testis. From

TABLE 7

SIZE OF PROSTATE IN RELATION TO BODY WEIGHT AND SPERMATOZOA

Size of Prostate	Weight Class of Rat (Gm.)						Spermatozoa		
	0–99	100–199	200–299	300–399	400–499	500–599	None	Few	Many
Large	1	1	19	73	55	4	2	2	148
Medium	0	0	36	66	19	1	0	1	121
Small	220	135	57	10	1	1	278	36	90

TABLE 8

WEIGHT OF COWPER'S GLAND IN RELATION TO BODY WEIGHT AND SPERMATOZOA

Cowper's Gland Weight (Gm.)	Weight Class of Rat (Gm.)						Spermatozoa		
	0–99	100–199	200–299	300–399	400–499	500–599	None	Few	Many
0.00–0.09	226	144	41	.....	.....	.....	279	35	7
0.10–0.19	.....	1	16	1	1	.....	.....	1	17
0.20–0.29	.....	1	20	21	22	.....	.....	1	43
0.30–0.39	.....	.....	19	43	12	.....	1	2	71
0.40–0.49	.....	1	14	39	19	1	.....	.....	73
0.50–0.59	.....	.....	2	25	26	5	1	.....	58
0.60–0.69	.....	.....	2	8	8	.....	.....	.....	18
0.70–0.79	.....	.....	.....	2	3	1	.....	.....	6
0.80–0.89	.....	.....	.....	.....	1	.....	.....	.....	1

Table 9 it is evident that the error is only a small percentage of the total number of cases. This accuracy, coupled with the simplicity of observation, makes the visibility of tubules a good criterion of the presence of sperm for use in the field.

Neither the color of the testes (pearl, gray, black) nor the presence of branches to the spermatic artery is correlated with the presence of sperm.

CRITERIA OF AGE AND SIZE AT MATURITY

The determination of the age of rats is a complex problem. Knowledge of the relation between chronological age (days or weeks) and physiological age (weight, length, etc.) is at best scanty. Studies of the growth of wild rats in nature and in cages (Davis and Winsor, 1948) show that the rate of growth varies greatly among individuals. Nutrition and position in the social hierarchy are among the important factors. Because of the complex nature of this problem it is possible merely to present correlations and indicate the value of certain morphological characters.

The relation between length (body plus head) and weight of rats (Fig. 3) is not a straight line. From the shape of the curve it is clear that rats continue to gain weight after the increase in length has practically ceased. Hence weight is considered to be better than length as a measure of age and is used in preceding sections of this paper.

To compare ages of individual rats, several characters are available. The eruption of the third molar may be compared with the body weight (Table 5). From these data it can be calculated that the weight at which 50 per cent of the rats have the third molar erupted is 67 ± 17 gm. The ossification of the skull may also be compared with the weight (Table 5). The weight at which 50 per cent of the rats have ossified skulls is 92 ± 20 gm. Although the spread is great, it may be concluded that in rats the eruption of the third molar occurs at a somewhat smaller size (earlier age) than does ossification of the skull. Thus, for example, a rat with the third molar not erupted and the skull not ossified is likely to be younger than a rat with the third molar erupted but the skull not ossified. Similarly, the latter rat is younger than a rat with the molar erupted and the skull ossified.

The sexual organs provide measures of age as well as of sexual maturity. The relation between weight and position of the testis is shown in Table 5. The weight at which 50 per cent of the rats have scrotal testes is 105 ± 45 gm.

The presence of sperm in relation to weight is shown in Table 4. For the purposes of calculation of maturity, the rats with "few" spermatozoa are grouped

TABLE 9

RELATION OF LENGTH OF TESTES AND VISIBILITY OF TUBULES TO SPERMATOZOA

Spermatozoa	Length of Testis (Mm.)					Tubules	
	0.05–0.09	0.10–0.14	0.15–0.19	0.20–0.24	0.25–0.29	Visible	Not Visible
None	201	73	4	2	0	5	273
Few	1	14	21	4	0	8	31
Many	1	7	40	253	59	308	32

with rats with "none," since such rats probably are not yet fertile. The weight at which 50 per cent of the rats have "many" spermatozoa was 200 ± 70 gm.

The succession of physiological characters for relative ages, therefore, is as follows: eruption of third molar (67 gm.); ossification of the skull (92 gm.); descent of the testes (105 gm.); presence of sperm (200 gm.).

DISCUSSION

The data presented in this paper show that there is no change in the reproductive state of the male Brown Rat from season to season in Baltimore, Maryland. These rats, of course, lived indoors, but a sample of 1,422 males collected in backyards in Baltimore also lacked seasonal changes, although many of them lived exclusively outside of houses. The male Brown Rat, therefore, appears to contrast strikingly with other rodents (Asdell, 1946; Hamilton, 1941; Rowlands, 1936).

It should be noted that the rodents of the family Muridae thus far studied contrast with the rodents of the family Cricetidae. Male Brown Rats, Roof Rats, Multimammate Mouse (Brambell and Davis, 1941), and probably male House Mice (Laurie, 1946) are fertile at all seasons of the year. In contrast, male Voles of three species, male Muskrats, and apparently males of several other species of Cricetidae (Asdell, 1946) have a nonfertile period of testes regression. It is entirely possible that these two families differ fundamentally in this reproductive aspect.

The possibility that young rats become sexually mature at a lighter weight (younger age) in some seasons was considered. No indication was found that rats produce sperm at a lighter weight or that the secondary sex organs develop more rapidly in the spring or summer than in the winter. There is an indication (not statistically significant) that in the summer the testes descend into the scrotum at a lighter weight of rat than in the winter; but this correlation, if true, could be purely a temperature relation.

No evidence of reproductive senility as judged by number of spermatozoa in the testes was found in even the large rats, although two cases of pathological testes occurred.

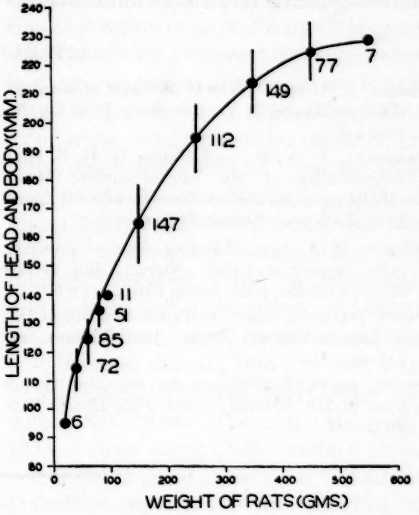

FIG. 3.—Relation between weight and length of rats. Numbers represent total rats in weight group. Vertical lines represent 1 standard deviation on each side of mean weight.

SUMMARY

An investigation of the reproductive organs of male Brown Rats was made to determine whether seasonal changes occur and to develop simple field criteria of reproductive activity.

A total of 721 male Brown Rats was collected in poultry warehouses in Baltimore, Maryland, from January 1, 1946, to March 3, 1947.

There is no evidence of seasonal changes in the size of the testes, the seminal vesicles, ventral prostate, or

Cowper's gland or in the presence of sperm.

A satisfactory field criterion for activity of the testes is visibility of the seminiferous tubules through the tunica albuginea.

Several characters may be used to indicate relative ages of rats. The weights at which 50 per cent of the rats have the following characters are as follows: eruption of third molar (67 ± 17 gm.); ossification of the skull (92 ± 20 gm.); descent of the testes (105 ± 45 gm.); presence of sperm (200 ± 70 gm.).

It is concluded that male rats living in a sheltered environment in Baltimore, Maryland, are fertile throughout the year. Seasonal changes in reproductive rates apparently depend upon the female.

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THE OCCURRENCE OF INCREASED ACTIVITY OF THE THYROID GLAND IN RAINBOW TROUT AT THE TIME OF TRANSFORMATION FROM PARR TO SILVERY SMOLT

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THE young rainbow trout of certain Michigan streams exhibit a characteristic migratory behavior. Usually after about two years spent in their native stream, these trout collect in the lower reaches, where they undergo a striking change in color and surface markings. The dark bars and spots of the juvenile or parr are replaced by a bright silvery sheen, which extends from well above the lateral line to the belly. The back remains a dark-green color, the black spots persist, while the dorsal and caudal fins become darker. Following this color transformation, which occurs dur-

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ing the fall or spring months, the silvery trout or "steelhead," as they are commonly called, migrate into one of the Great Lakes. The occurrence of this phenomenon in rainbow trout has been described by Greeley (1933), who considers it analogous to the change from the parr to the smolt phase of the salmon. He found that some rainbow trout in Michigan remained in the stream until mature, presumably throughout their lives, and that such trout did not pass through the silvery color change. Furthermore, in certain streams the nonmigratory behavior predominated, while in others the majority of the trout were migratory in nature. In discussing the taxonomy of the rainbow trout in Michigan waters, Greeley quotes Hubbs, who states that both *irideus* and *shasta* forms have been propagated in Michigan with more or less crossing in hatcheries, but that *irideus* predominates in the waters. Hubbs (1929) regards both forms as subspecies of *Salmo gairdnerii*, the Columbia River steelhead. These Pacific Coast trout migrate to the sea as silvery smolts.[2]

The color transformation, characterizing the transition from parr to smolt, is of particular interest from both physiological and ecological points of view, since it represents a marked alteration in certain bodily functions suggestive of preparation for a change in habitat. It appears to be a phenomenon common to all the young salmonoid fishes which migrate from fresh water to the sea after having spent a year or more in their native water. Even the brook trout, a characteristically nonmigratory species, when it does enter salt water changes to a silvery color, as described by White (1940).

[2] Neave (1944) believes that he has been able to identify two separate races of *S. gairdnerii* in the Cowichen River system of Vancouver Island, one migratory and one nonmigratory.

There is surprisingly little information available concerning the nature of the color transformation from the dark parr to the silvery smolt. While it is generally recognized that the silvery color of fish is due to guanine crystals either within special cells called "iridocytes" (guanophores) or deposited in layers in the skin, the author has been able to find no observations in the literature on the distribution of guanine in parr and smolt stages of the salmonoids. Likewise, data on changes in chromatophores of the skin during this stage of development are lacking. However, some study has been made of certain other physiological changes occurring in the smolt. Lovern (1934) found alterations in their fatty acids, which exhibited a chemical constitution intermediate between those of fresh-water and those of salt-water fishes; and Huntsman and Hoar (1938) observed increased resistance of salmon smolt to salt water as compared with the parrs. The most significant investigation in this field is that of Hoar (1939), who found a greatly increased thyroid activity at the time of transformation from the parr to the smolt stages in the Atlantic salmon.

The present investigation comprises a study of the alterations in the skin responsible for the color transformation of the young rainbow trout, together with associated changes in the secretory activity of the thyroid gland.

SOURCE OF MATERIAL

The trout were obtained from two streams of the northern Michigan peninsula. Most of them were taken from the Black River, which empties into the northernmost part of Lake Michigan. A few were caught in the Two Hearted River just above its outflow into Lake Superior. The trout were secured almost entirely by angling; several were caught in gill nets. The latter method is unsatis-

factory for studying coloration, since, in addition to injury of the skin, contraction of the melanophores occurs with asphyxia and death. The parrs were secured through two seasons, 1946–47, from early April until late September. The silvery smolts were taken during August and September in the Black River and only in the half-mile stretch above the mouth of the stream. In the Two Hearted River smolts were caught early in June. The present study consists of observations on twenty-five juvenile or parr trout aged two to three years and eight silvery smolt, one to three years of age, as well as two trout in the process of transformation from parr to smolt (Tables 1 and 2).

METHODS OF STUDY

a) Photography.—Photographs of the live fish were made while they were under ether anesthesia (1 ounce of ether to the gallon of water). The anesthetized fish was fastened by means of soft metal alloy (soldering) wires to a tilting board in a small aquarium, which made it possible to secure the desired angle of incident daylight. Care was taken to avoid deep anesthesia, which causes contraction of the superficial melanophores, with consequent pallor. With light anesthesia the color does not change.

b) Observations of the intact fish under the microscope.—It was found possible to determine the state of contraction of the skin melanophores by direct examination with a dissecting microscope either very briefly by holding the fish in a damp cloth or under light ether anesthesia, which allowed much more careful inspection, or immediately after killing by a sharp blow on the head. Following instantaneous death by this means there is no change in the state of melanophore expansion for some time. It is necessary prior to examination that the fish be kept on a background of the same general shade as that of its natural environment, since light backgrounds cause melanophore contraction.

c) Preparations of cutaneous and subcutaneous melanophores.—In order to secure satisfactory specimens of skin melanophores for permanent mounts, vertical strips of skin were removed immediately after the fish had been killed and were placed in 10 per cent formalin solution for 5–10 minutes. Next, the strips were dehydrated for 1 hour in 95 per cent ethyl alcohol, followed by two hourly changes in absolute alcohol, and then were placed in terpineol overnight. Very viscous balsam or damar dissolved in benzene was employed as the mounting medium, and, on account of the tendency of the skin to curl, weights were placed on the cover slips.

Preparations of the subcutaneous tissue, which contains the melanophores forming the parr marks and a layer of guanine crystals, designated as the "argentum," were made by including in the excised tissue a layer of underlying muscle. This material was treated in the same manner as the skin strips except that, before mounting, the muscle was scraped off with a safety-razor blade. Specimens of the operculum were also dehydrated in alcohol and terpineol.

d) Preparations of guanine crystals.—The most satisfactory method found for preserving the layer of guanine crystals on the scales was to place skin strips in glycerin and keep them in the icebox. Specimens of the whole skin could then be mounted in glycerin-gelatin medium at any subsequent time. To secure individual scales, the skin strip was transferred to 0.9 per cent NaCl solution for a few hours (at icebox temperature); then, under a dissecting microscope, scales could be removed from their pockets by means of a sharp-pointed dissecting needle without disturbing the guanine crystal layer. Before the glycerin-gelatin mounting medium was used, the excess glycerin was removed with filter paper.

The other chromatophores, namely, erythrophores and xanthophores, present in certain areas of the trout's skin are well preserved in the glycerin-gelatin mounts.

e) Autopsy.—Post-mortem examination included determination of weight, length, sexual maturity, age from scales, as well as removal of skin strips, operculum, thyroid, and other organs, including pituitary, pancreas, and gonads, for further studies. Material for histological preparation was fixed in Bouin's solution. Sections for thyroid tissue were taken through the first, second, and third basihyals or copulae from each fish.

NATURE OF COLOR CHANGE

The difference in appearance between typical parr and silvery smolt is shown in Plate I, Figures 1 and 2. Both were two-year-old trout and were caught in the

TABLE 1

RAINBOW TROUT PARRS

Trout No.	Date Killed	Length (Inches)	Weight (Ounces)	Sex	Age in Scale Annuli	Present Season's Scale Growth Rings	Average Height of Secreting Cells of Thyroid Gland (μ)
1	5/2/46	7 1/8	2	♂	II	1	8.32
2	5/2/46	6 1/2	1 1/2	♀	II	1	7.27
3	5/2/46	8	3	♂	II	3	6.64
4	5/4/46	6	*	*	II	*	6.93
5	5/4/46	8 3/4	4	♂	III	1	3.71
6	5/6/46	7 3/4	*	*	II	1	5.81
8	5/11/46	6 3/4	2	♂	II	1	4.62
9	6/23/46	7 1/2	*	*	II	5	5.22
10	6/23/46	8 1/2	3	*	II	4	5.54
11	6/23/46	6 3/4	*	*	II	5	5.77
14	7/7/46	7 3/8	2	♀	II	5	5.25
22	8/7/46	8	*	*	II	9	3.47
23	8/7/46	9	*	*	II	9	3.97
29	8/24/46	7 1/4	*	♀	II	*	5.39
33	8/30/46	8 1/2	*	*	II	*	6.69
55	10/10/46	8	*	♀	II	*	5.22
73	6/30/47	7	*	♂	II	7	4.71
93	8/3/47	10	4 1/2	♂	III	5	5.51
95	8/5/47	6 3/4	1	♂	*	*	5.28
105	8/25/47	7 5/8	2	♀	II	11	4.75
109	8/20/47	8 3/8	3	♀	II	15	5.22
110	8/30/47	5 5/8	1	♂	II	*	5.65
114	9/2/47	7	1 1/2	♀	II	8	5.18
118	9/11/47	7 1/4	*	♀	II	11	4.55
127	9/21/47	6 3/4	1 1/2	♂	II	7	5.60

* Not determined.

TABLE 2

RAINBOW TROUT SMOLTS

Trout No.	Date Killed	Length (Inches)	Weight (Ounces)	Sex	Age in Scale Annuli	Present Season's Scale Growth Rings	Average Height of Secreting Cells of Thyroid Gland (μ)
	A. Silvery Smolts						
69	6/28/47	7 3/4	3	♀	II	7	8.99
70	6/28/47	8 1/8	3	♂	II	7	10.97
71	6/28/47	8 1/4	4	♂	II	7	9.48
97	8/10/47	8 3/4	3 1/2	♂	I	14	7.74
112	9/3/47	10 1/4	6	♀	II	18	10.55
113	9/3/47	10 1/4	5 1/2	♂	III	8	8.59
115	9/4/47	12 3/8	11	♀	II	23	5.54
117	9/9/47	11 3/4	9	♀	II	22	6.80
	B. Transforming Smolts						
86	7/21/47	7 1/2	2 1/2	♂	II	8	8.26
103	8/25/47	5 3/8	1	♂	II	7	8.11

same area of the stream in early September, 1947. The parr was $7\frac{1}{4}$ inches long, while the smolt measured $10\frac{1}{4}$ inches. Comparison of the scales of these two fish showed 11 circuli distal to the second annulus in the case of the parr and 18 such circuli on the scales of the smolt, indicating much more rapid growth of the latter trout. As shown in Tables 1 and 2, the smolts of the same age and date of capture were, on the average, larger than the parrs and exhibited a greater number of new growth rings. It will be noted in Plate I, Figure 1, that the six or more oval dark parr marks situated along the region of the lateral line and the spots on the side are not visible in the smolt (Pl. I, Fig. 2).[3] The narrow red stripe not infrequently present along the lateral line of the parr also disappears with the color transformation. The operculum of the smolt is a bright silvery hue and contrasts sharply with the dark dorsal head area.

Stripping off the skin of the silvery fish revealed an underlying pearl-gray to silvery layer—the argentum—which in most instances appeared to be devoid of pigmentation (Pl. I, Fig. 6). In several of the smolts one could detect faintly pigmented areas in the argentum, suggesting faded parr marks. Removal of the skin from the parrs, on the other hand, brought out an intensification of the black pigmented areas seen less distinctly in the intact fish (Pl. I, Fig. 5). The subcutaneous layer, or argentum, if it can be so designated, in the parr was a dark to pearl-gray.

This alteration in color and surface markings characterizing the transformation from parr to smolt was found to be due to two changes occurring in the skin and subcutaneous tissues, namely, an increased deposition of guanine crystals and the contraction and disintegration of melanophores in certain areas. These changes will be described in some detail because they constitute the only manifestations of increased thyroid activity which have been recognized thus far in the young rainbow trout.

GUANINE

Examination of the detached scales of the rainbow trout under the microscope discloses a number of long hexagonal crystals arranged transversely to the long axis of the scale and applied closely to its medial surface (Pl. I Fig. 7). These crystals give the chemical reactions for guanine[4] and, when observed with transmitted light, exhibit the various colors of the spectrum, all of which are sometimes seen in a single crystal. The color of the crystals is due to diffraction of light caused by superimposition of several crystals. Single ones are colorless. When illuminated from above, they exhibit a silvery sheen. Careful examination with various magnifications and different kinds of illumination has failed to reveal any suggestion of a cell membrane or nucleus associated with the individual or group of crystals. The only possible suggestion of structure lay in the finding that, when the guanine crystals were detached from the scale, they tended to come off as a unit, though individual crystals and groups of crystals separated easily from the layer. Very slight pressure of the dissecting needle was required for this purpose.

When the scales of parr and silvery smolt were compared, a marked difference in the quantity of guanine deposited

[3] The exposure was essentially the same in the two photographs, as is evident by the shade of the background.

[4] The crystals are insoluble in water, alcohol, ether, chloroform, and acetic, formic, and lactic acids, and ammonia but dissolve in mineral acids and hot ammonia (Millot, 1923).

on them was observed. In the parr (Pl I, Fig. 3) the crystals were present in a relatively sparse layer, confined to the distal or caudal half of the scale. The circuli were visible through them. Scales of the smolt, on the other hand, exhibited a dense layer of guanine (Pl. I, Fig. 4), which obscured the circuli and often extended beyond the center of the scale onto the cephalic half. These differences could be observed well, both grossly and microscopically, in the mounted specimens of skin, especially those in glycerin-jelly, which preserves the colors of the crystals. Such strips of the parr's skin exhibit, with reflected light, a faint bluish hue, while the smolt's skin strip reflects a bright silvery sheen. Microscopically, it was seen that the quantity of guanine crystals on the scales of the parr varied greatly—many of the scales appeared to have few or no crystals on them, while every scale in the silvery zone of the smolt's skin was seen to possess a heavy mat of guanine. The deposition of crystals was greatest from just above the lateral line to near the belly. Scales of the dorsum and ventral surface showed relatively little guanine.

The same relative difference in guanine content between parr and smolt was exhibited by the subcutaneous layer. In the smolt the layer of guanine was so heavy as to obscure the melanophores when the preparation was observed by reflected light. The thickness of this layer increased from the lateral line ventrally, and at the belly it had a milky-white or chalklike appearance. Figures 5 and 6 (Pl. I) show the difference between the parr and the smolt with respect to the visibility of parr marks. The operculi of the smolts likewise were found to contain very much denser accumulation of guanine crystals than did those of the parrs.

MELANOPHORES

With a view to determining what role the melanophores played in the loss of surface markings in the smolt, a detailed comparison was made of the distribution and state of contraction of the melanophores in parr and smolt. Observations were carried out on the intact fish both during life and immediately after death, as well as on preparations of the skin, subcutaneous tissues, and operculum. Aside from its silvery flanks, one of the most striking differences between the smolt and the parr is the white belly of the former. The dark-gray belly of the parr is due to a relatively high concentration of fully expanded melanophores in the skin of this region. Examination of the corresponding region of the smolt revealed a marked diminution to complete absence of melanophores, and the remaining ones were in a state either of partial contraction[5] or of actual disintegration. Figures 8 and 9 (Pl. II) are photomicrographs of the skin of a parr and a smolt taken from identical regions of the ventral surface. The melanophores of the lower flanks of the smolt were observed to be usually partially contracted; but in the remainder of the skin these pigment cells were as fully expanded as in the parr.[6]

When viewed by transmitted light, the melanophores of the smolts' argentum appeared to differ little in number or state of expansion from those of the parr. In one instance only was evidence of disintegration observed. On the other hand, the operculi of the silvery fish showed a

[5] The customary usage of "contraction" and "expansion" is employed here instead of the more accurate designation of "dispersion" and "concentration."

[6] It was found impossible to employ Hogben and Gordon's (1930) melanophore index because of the wide variations in degree of expansion exhibited by trout melanophores within a small area.

considerable proportion of these pigment cells to be in a state of partial contraction, and many showed evidence of disintegration. The operculum melanophores of the parrs were, for the most part, fully expanded, though local areas of partial contraction were observed in some cases. No evidence of disintegration was seen.

The capture of two trout in the process of color transformation provided a valuable link between the parr and the smolt. These fish exhibited a pinkish metallic sheen. In one the parr marks were not visible; in the other they were distinct but faint. The belly of one was dark, the other almost white. Examination of the detached scales showed a considerable increase in the density of the guanine crystal layer over that of the parr but not so heavy as that exhibited by the silvery smolts. In the fish with the dark belly the melanophores were fully expanded, while in the other with the pale ventral surface melanophores were very sparse and slightly contracted. When the skin was stripped off, heavily pigmented parr marks on a dark-gray background were seen in both fish. The opercula exhibited melanophores in a state of slight focal contraction to full expansion.

The absence of visible parr marks in one of these two trout provides evidence that the layer of guanine crystals on the scales is, by itself, sufficient to account for the loss of this juvenile characteristic. When this layer of guanine is sufficiently thick, it appears to act as a mirror, preventing light from reaching structures in the dermal and subcutaneous tissues.

OTHER CHARACTERISTICS

Erythrophores and xanthophores were present in the skin of both parrs and smolts but were obscured in the latter by the thick layer of guanine crystals on the scales. These chromatophores could be seen in the mounted skin strips of the smolt when viewed from the medial surface.

The muscle of all the parrs was white, as was that of the three smolts taken in the Two Hearted River, while all five smolts secured in the Black River had salmon-colored flesh. Silvery smolts taken after midsummer were considerably larger than the parrs of the same or greater age and showed, on the average, many more circuli of the present season's growth (Tables 1 and 2). All the trout included in this study were immature sexually and exhibited very small gonads.

ACTIVITY OF THE THYROID GLAND

Excellent descriptions of the thyroid gland of the salmonoid fishes have been given by Marine and Lenhart (1910), Gaylord and Marsh (1912) for the brook trout, Guternatsch (1910) for the rainbow trout, and Hoar (1939) for the Atlantic salmon. The essential structure is the same in all three forms and consists of follicles diffusely arranged about the ventral aorta and extending from the first to the third branchial arches. The fibrous capsule characteristic of the mammalian thyroid gland is lacking. Histologically, the individual follicle resembles closely that of the mammal except for the fact that the cell boundaries, especially in the resting gland, are less distinct. Hyperplasia is characterized by increase in the height of the secreting cells, vacuolization and loss of intrafollicular colloid, invagination of the walls of the follicles, and increased vascularity of the gland.

PARR

The typical appearance of the thyroid of a two-year-old rainbow trout parr is shown in Plate II, Figure 10. The follicles vary considerably in size, ranging from 0.01 to 0.2 mm. in diameter, and are com-

pletely filled with deeply staining acidophilic colloid. The cells, as shown in Plate III, Figure 11, are cuboidal or flattened, with rather indistinct cell boundaries, but they exhibit clearly defined nuclei and a homogenous cytoplasm. Vacuolated colloid was observed in some follicles and not in others; furthermore, the number of vacuoles varied from fish to fish.

In his study of the salmon parr, Hoar found seasonal changes in thyroid activity—greater activity in the spring and less in the summer and fall. In the present study of twenty-five rainbow parr taken from early spring to late autumn there was some evidence of a similar variation in activity of the thyroid, in that the highest follicular cells were found in trout taken in early spring.

SMOLT

The thyroid of the smolt differs markedly from that of the parr in a number of respects, all indicative of increased glandular activity. The most conspicuous changes are the transformation of the epithelial cells from cuboidal to columnar in form and the loss of colloid from the follicles. These differences can be easily detected in the low-power view shown in Plate III, Figure 12 and more strikingly seen in the photomicrographs made at higher magnifications (Pl. III, Figs. 13 and 14). The follicular epithelium, when examined under oil immersion, exhibited fine vacuoles and irregular or ragged distal surfaces, which are indications of actively secreting cells. Many of the follicles have lost their spheroidal shape, some show distinct invaginations and others partial to complete collapse of the walls, as seen in Figures 13 and 14 (Pl. III). These latter appear as cords of cells. The vascularity of the smolt thyroid was definitely increased, as shown by the large number of blood cells in the interfollicular lymph and blood channels (Pl. III, Figs. 12 and 13). This increased blood supply was reflected grossly by a pink coloration of the floor of the mouth over the area between the first and second branchial arches which was not observed in the parrs. The number of follicles in the thyroids of parrs and smolts of similar size did not seem to differ significantly, although this was a difficult matter to decide because of the considerable variation encountered between different areas of the thyroid in the same fish. However, in the large smolts, Nos. 112, 113, 115, and 117 (Table 2), there was a definite increase in the amount of thyroid tissue, made up largely of small and collapsed follicles.

The two transforming smolts, Nos. 86 and 103 (Table 2), showed definite hyperplasia of the thyroid; but evidences of glandular activity were not so marked as was seen in the most active glands of the silvery smolts, e.g., Nos. 70, 71, and 112. Since obvious individual differences in degrees of thyroid activity were apparent in both parrs and smolts, it seemed important to secure a quantitative estimate of the hyperplasia of these thyroids. Measurement of the height of the secreting cells, as described by Rawson and Starr (1938), was employed for this purpose. By means of an ocular micrometer at 480× magnification, the height of the representative (average) cell in each of 100 consecutive follicles was determined. The average of these measurements for each trout is shown in Tables 1 and 2 and Figure 15. It will be noted that more than half the parrs showed thyroid cells between 5 and 6 μ in height, the mean was 5.46 μ, and the median 5.30. The silvery smolts showed a range of cell heights of from 5.54 to 10.97 μ, the mean, including the smolts in the process of transformation, was 8.5 μ, and the median 8.43. The two

silvery trout, Nos. 115 and 117 (Table 1), exhibiting the lowest cell height of the smolt series, were considerably larger than the other fish and had probably undergone the transformation sometime earlier in the summer.

DISCUSSION

On the basis of the evidence provided by this study, it seems fair to conclude

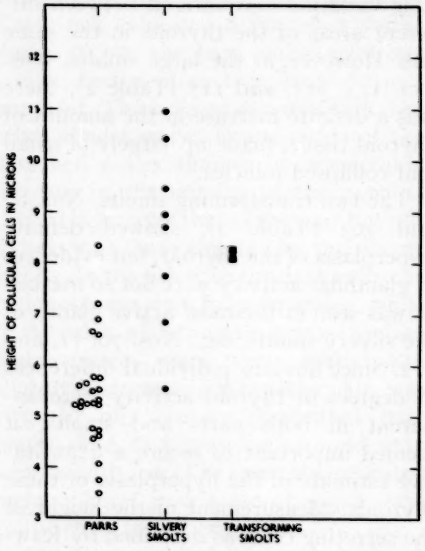

FIG. 15.—Comparison of heights of secreting cells in thyroids of parrs, silvery smolts, and transforming smolts. Each circle or square represents the average height of 100 cells measured in each fish.

that the degree of thyroid hyperactivity exhibited by the silvery smolt does not occur in the parr. With the exception of the two largest smolt, in which it is likely that the increased activity of the thyroid had subsided, and a single parr, the thyroids of all the smolts manifested greater functional activity than did any of the parr thyroids. This difference is best expressed quantitatively by comparison of the heights of the secreting cells (Fig. 15). It may be stated that the general histological features of these changes in the functioning of the thyroid gland of the rainbow trout are identical with those described by Hoar (1939) for the silvery smolt of the salmon.

Of the other differences between the rainbow parr and the smolt, by far the most conspicuous and constant is the greatly increased deposition of guanine, especially on the scales. That this layer of guanine crystals can produce the silvery sheen of the smolt's sides and the loss of surface markings, despite the presence of fully expanded melanophores, seems evident from the present observations. However, the pallor of the smolt's belly appears to depend on contraction and disintegration of the melanophores in this region, where the layer of guanine on the scales is relatively thin. The sparsity of guanine on the scales of the belly seems to be compensated for by the subcutaneous deposition of guanine crystals, which is heavier in this region than elsewhere. The silvery appearance of the operculum appears to be due more to a heavy guanine deposit than to contraction and disappearance of melanophores, although these changes in the pigment cells undoubtedly contribute to the color transformation.

Ever since the work of Gudernatsch (1913), who first demonstrated the important role played by the thyroid hormone in the metamorphosis of anurans, attention has been focused on the thyroid gland in those amphibians and fishes which pass through a larval stage in their development, and numerous instances of thyroid hyperactivity associated with metamorphosis have been reported. The principal contributions to this subject in fishes are those of Sklower (1928) and Hagen (1936), who described marked hyperplasia of the thyroid gland in the larva of the eel at the time of transforma-

tion from leptocephalus to elver. Sklower (1930) found a similar phenomenon occurring in the metamorphosis in the flatfish (*Pleuronectes platessa*). Harms (1929) states that the thyroid controls metamorphosis in the goby, *Periophthalmen* and *Boliophthalmen*, and in the blenny, *Salarias*. He described alteration in the distribution of thyroid follicles, which become more concentrated at metamorphosis and show certain changes in the colloid that are characteristic of the adult gland. No mention is made of hyperplasia. He did find, however, that metamorphosis was greatly accelerated in these species by feeding them thyroid powder.

The question arises as to how the transformation from parr to smolt in the salmonoid fishes should be classified. Hoar (1939) regards this change as a true metamorphosis, as does Huntsman (1948). These authors would include, in addition to color change,[7] the alterations in constitution of body fatty acids and increased tolerance to higher salinities of water, mentioned earlier; but they describe no definite changes in morphology as distinguishing the smolt from the parr. Since the only constantly observed differences between the rainbow parr and the smolt were physiological alterations in the guanine metabolism and local changes in melanophores, it would not seem justifiable, at the present stage of our knowledge, to designate this transformation as "metamorphosis."

Some of the rainbow smolt exhibited sufficiently striking differences from the parr to suggest the possibility that the thyroid hormone might be producing other effects. Could the very rapid growth of the silvery smolts, Nos. 97, 112, 115, and 117 (Table 2), as compared with similar-age parr caught in the same part of the stream, be attributable to an increased metabolic or growth-producing function? Grobstein and Bellamy (1939) fed thyroid powder to immature *Platypoecilus maculatus*, with resulting diminished rate of growth. Smith and Everett (1943) found no growth-accelerating effect of thyroid hormone administered to *Lebistes reticulatus* in food or water, nor was their oxygen consumption increased. Elkin, Root, and Mofshin (1940) fed thyroid powder to goldfish, and Hasler and Meyer (1942) injected thyroxin into the same species and observed no increase in metabolic activity as measured by oxygen uptake. These latter authors incline toward the view that the thyroid in cold-blooded vertebrates has more to do with affecting specific tissue responses, such as metamorphosis, than it has with regulation of the metabolic rate. On the other hand, Goldsmith and associates (1944*a*, *b*) found growth retardation of three different fishes, including goldfish and *P. maculatus*, when they were kept in water containing thiourea, which interferes with the normal functioning of the thyroid gland. Since the fishes exhibited no deleterious effects other than that on the thyroid, they believe that their results provide presumptive evidence that the thyroid gland is concerned with growth in fishes. It seems not improbable that the apparently divergent results of these several groups of investigators may be due to the fact that certain of them were measuring response of fish to increased quantities of mammalian thyroid hormone, while others observed the effect of depriving the fish of its own thyroid secretion. No studies of this nature on salmonoid fishes have been reported. It is, of course, possible that the large silvery smolts of the present study had migrated to Lake Michigan and had returned to the stream and that the more

[7] Hoar (1939) states in passing that the seaward-migrating salmon smolt is covered with guanine crystals.

abundant food supply in the lake accounted for the rapid growth. Examination of their scales showed "migration" checks[8] following the last annulus in several of them, which lends some support to this possibility. However, the largest one showed no such mark on the scales. Acceleration of growth may well be due to increased production at this time of the growth-promoting hormone by the pituitary. At any rate, it is evident that much more information is necessary as to both actual growth rates, movements of the smolts, endocrine studies, etc., before even tentative conclusions on this subject can be drawn.

Another distinctive characteristic of the Black River rainbow trout smolts was their salmon-colored flesh, a feature which none of the parr exhibited, even those of greater age, caught in the same water. The chemical studies of Newbigin (1900) and Bailey (1936) and feeding experiments by André (1926) point to certain types of food, especially crustaceans, as the source of the carotenoid pigments giving rise to the salmon color of the muscles and the red and orange pigments in the skin. Since it is not known whether the smolts in this study had had access to the lake food, one can draw no inferences as to the etiology of change in color of flesh.

One can only speculate about the ecological import of the silvery color assumed by the rainbow trout smolt. That it may be of value in a large body of deep water as protection against predatory fishes seems not unlikely. To acquire visual information on the significance of the silvery reflection of fish scales, a German investigator, Kapelkin (1907), made observations from the bottom of a lake. Looking up through the water, he saw a clear or light-colored circle above his head. When the wind blew, the appearance changed. The whole surface was covered with shifting silvery stripes produced by the waves, which he described as looking much like darting, silvery-bellied fish. He concludes that the silvery hue affords protection, just as does the dark back of the fish on the background of a rocky bottom. In further support of this inference, he alludes to the very dark color of deep sea fish which live below the penetration of light. More direct evidence of the protective value of coloration was produced by Sumner (1935), who placed equal numbers of light-adapted and dark-adapted mosquito fish (*Gambusia patruelis*) in a light-colored tank, together with two penguins. Twice as many dark-colored fish as pale ones were eaten. On the other hand, when the same combination of fishes was placed in a dark tank, the penguins ate three times as many pale fish as they ate dark ones.

In the foregoing discussion of the changes occurring in the transformation of rainbow trout parr to smolt, a causal relationship between increased thyroid activity and the factors producing the silvery color has been implied. However, no certain conclusions can be drawn in the absence of direct experimental proof that the thyroid hormone is capable of affecting the deposition of guanine and bringing about alterations in the state and number of the melanophores. Evidence that the thyroid hormone does possess these functional activities will be presented in forthcoming communications.

SUMMARY

A study was made of the nature of the color transformation and associated changes in the secretory activity of the

[8] I am much indebted to Dr. John R. Greeley, of the New York State Conservation Department, for the interpretation of these scales. He suggests that what he has termed a "migration" check might represent interruption of growth during transformation from parr to smolt; but no data are available concerning growth during this stage of the trout's life.

thyroid gland which young rainbow trout in certain Michigan streams exhibit before migrating into one of the Great Lakes. This change in color from the relatively dark parr to the silvery smolt was found to be due principally to the deposition of a greatly increased amount of guanine on the internal surface of the scales, laid down as a dense mat of crystals. Contraction and disintegration of the dermal melanophores were observed to constitute an important part of the transformation process, but this change was observed only in the ventral surface and the operculum.

Examination of the thyroid glands of the rainbow trout smolts revealed markedly increased functional activity as compared with the parr thyroids. This was shown by change from cuboidal to columnar type of follicular epithelium; vacuolization and loss of colloid from the lumen of the follicles, which were distorted in shape and often collapsed; increased vascularity of the gland; and a greater amount of thyroid tissue. Measurement of the height of the follicle cells in twenty-five parrs and ten smolts gave an average height of 5.46 μ for the parrs and 8.5 μ for the smolts.

The character of the thyroid hyperactivity observed in the recently transformed rainbow trout smolt appears to be identical with that described for the smolt of the Atlantic salmon. The implications of these findings and their relationship to true metamorphosis occurring in certain fishes is discussed.

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PLATE I

FIG. 1.—Parr rainbow trout, two years old. Note dark oblong parr marks and heavy pigment spots characteristic of the juvenile trout.

FIG. 2.—Silvery rainbow trout smolt, two years old. Surface markings have disappeared.

FIG. 3.—Scale of rainbow parr showing sparse layer of guanine crystals, through which the circuli are visible. A few melanophores on the distal or posterior end of the scale. Magnification ×60.

FIG. 4.—Scale of silvery smolt, showing heavy mat of guanine crystals which obscures circuli and melanophores. Crystals absent on the anterior half of scale. Magnification ×33.

FIG. 5.—Subcutaneous layer of melanophores and guanine crystals of juvenile trout (Fig. 1), showing characteristic aggregation of melanophores to form parr mark. Magnification ×1½.

FIG. 6.—Subcutaneous layer of silvery smolt—melanophores are completely obscured by heavy layer of guanine crystals, so that parr mark is invisible. Appearance at this stage has given rise to term "argentum." Magnification ×1½.

FIG. 7.—High-power magnification of middle left-hand margin of Fig. 4, showing arrangement of hexagonal guanine crystals. Palest ones represent single crystals. Heaviness of shadows depends on number of superimposed crystals. Magnification ×960.

PLATE II

FIG. 8.—Skin taken from junction of flank and belly of rainbow parr No. 118, just posterior to pectoral fins, showing large numbers of fully expanded melanophores. Magnification ×40.

FIG. 9.—Skin of rainbow smolt No. 97, taken from area identical with that of parr No. 118 (Fig. 8), which shows complete disappearance of melanophores on belly surface (right-hand end of photograph); the few remaining melanophores are contracted and disintegrating. Magnification ×40.

FIG. 10.—Section through floor of mouth of rainbow trout parr No. 22 between first and second basihyals, showing arrangement of the thyroid follicles around the ventral aorta. The colloid-filled follicles, which exhibit a low cuboidal epithelium, are separated by fatty tissue, through which extends a network of vascular channels—blood vessels and lymphatics. Magnification ×90.

PLATE III

FIG. 11.—Thyroid gland follicles of rainbow trout parr No. 93, showing resting or inactive state characterized by cuboidal epithelium and abundant, deeply staining colloid, which exhibits relatively few vacuoles. Magnification ×360.

FIG. 12.—Thyroid gland of rainbow smolt No. 71, exhibiting marked hyperactivity—intense vascular engorgement, follicle cells high columnar; many of the follicles contain little or no colloid and are collapsed. Magnification ×100.

PLATE I

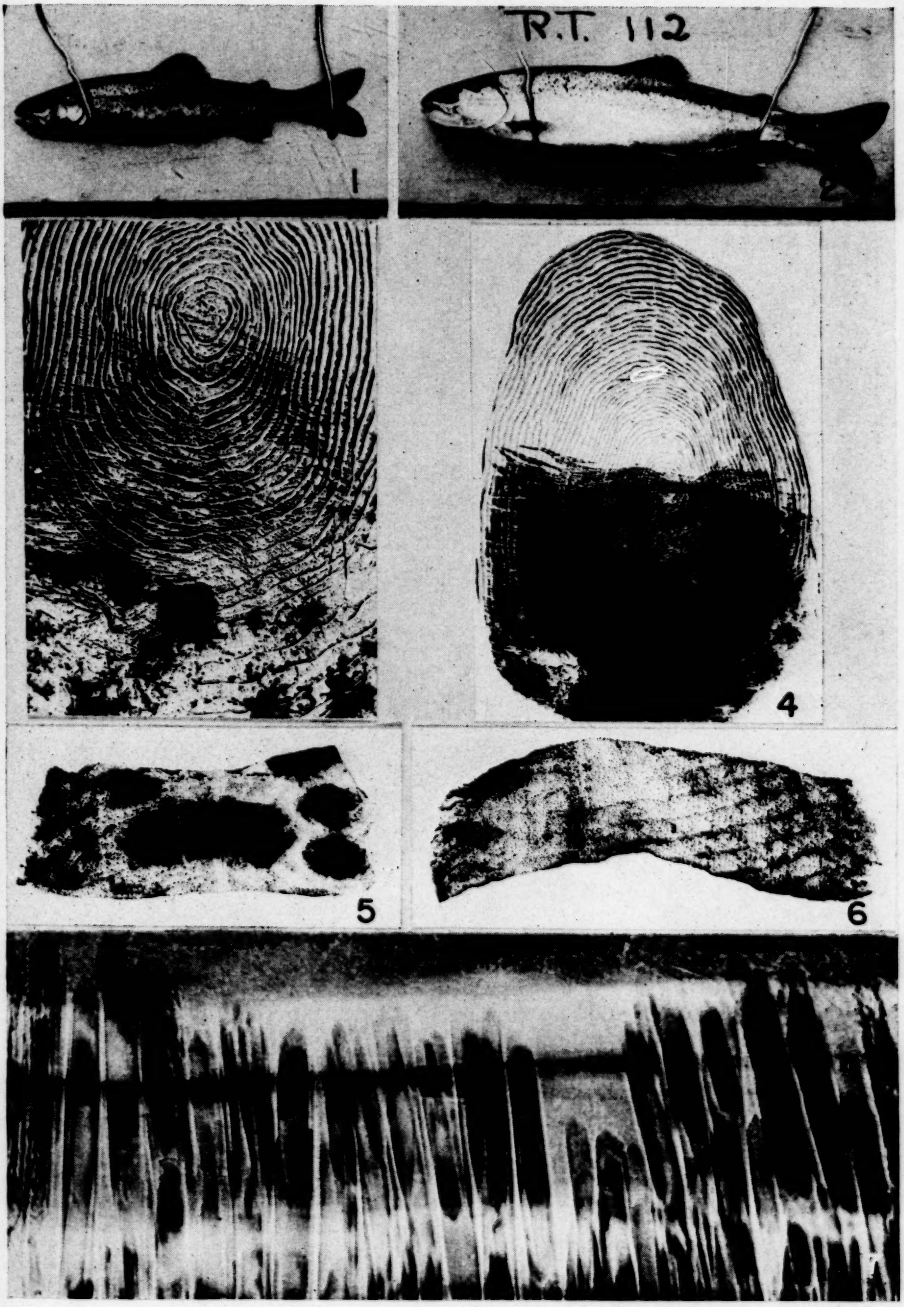

PLATE II

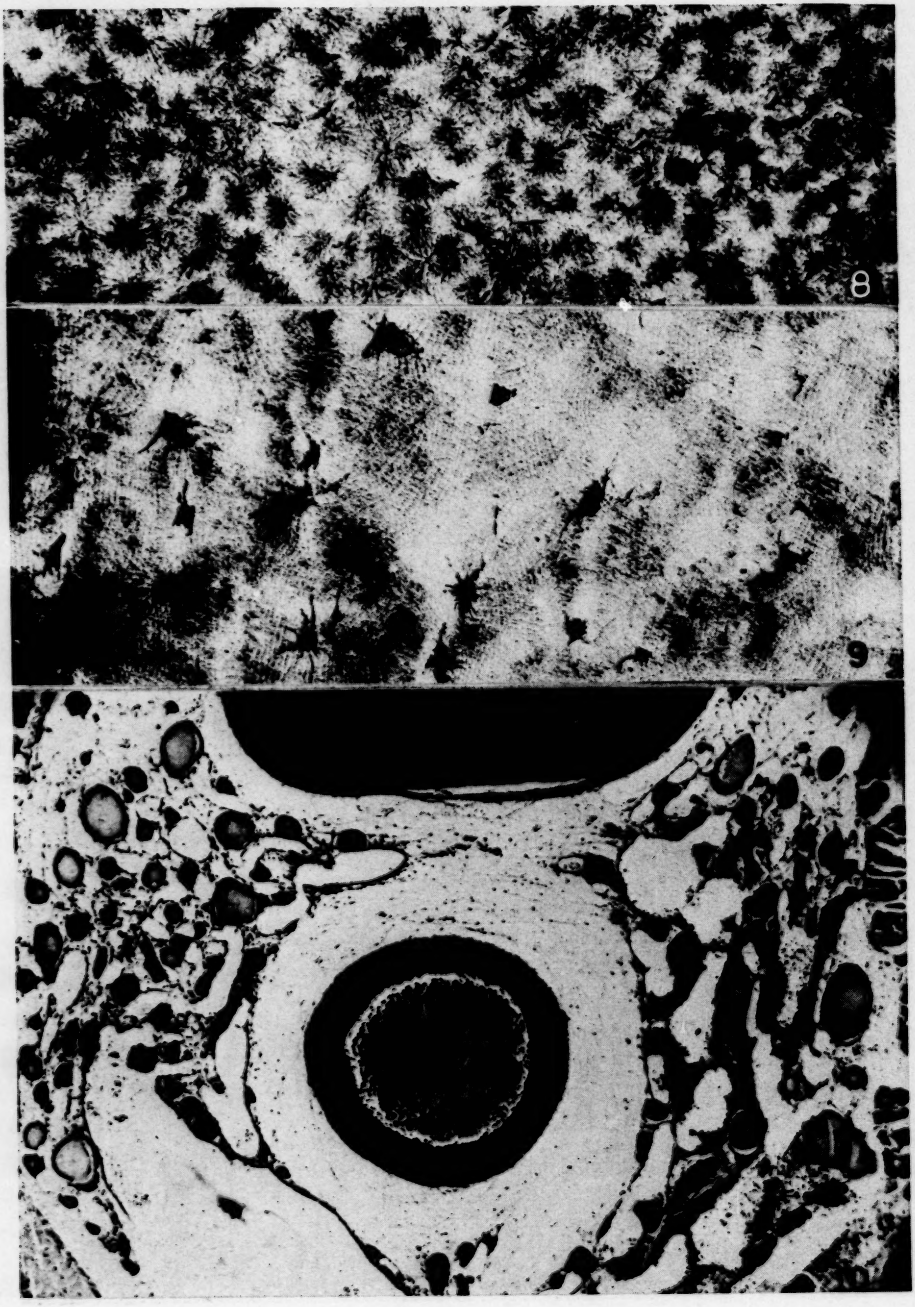

PLATE III

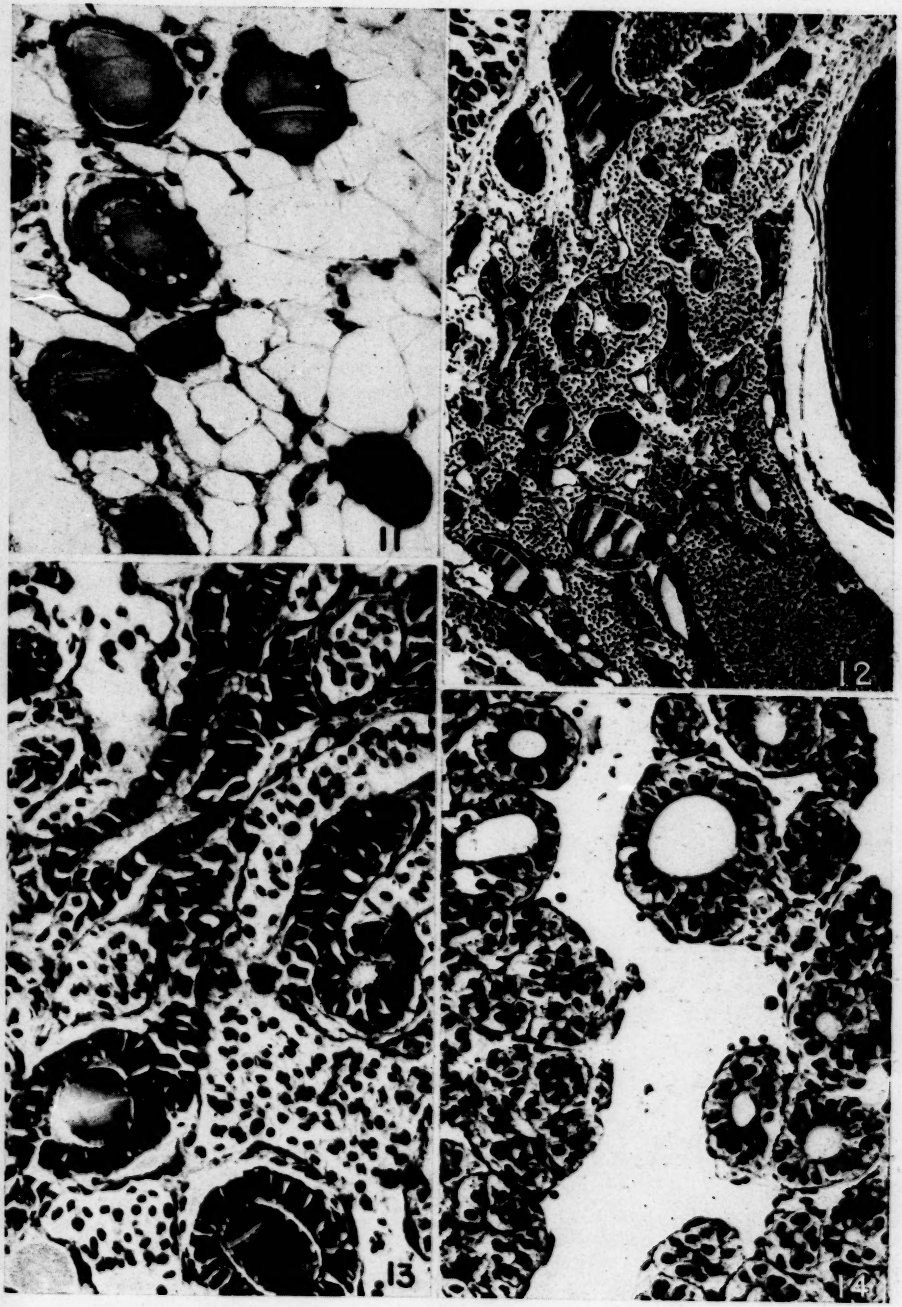

FIG. 13.—High-power view of central area of Fig. 12. The colloid remaining in the collapsed vesicles shows marked vacuolization. The nuclei of the secreting cells appear to occupy a large part of the cell. Magnification ×360.

FIG. 14.—Thyroid gland of rainbow trout smolt No. 112. Vascular channels empty. (Floor of mouth not pink, as in case of smolt No. 71, Fig. 13.) Follicles in not quite so active a secretory state as that shown in Fig. 12, although they contain relatively little colloid and the lumina are small. Magnification ×360.

ERRATUM

Part of the legend for Figure 2 in the article entitled "The Lipid Composition of *Tenebrio molitor* Larvae," by Asher J. Finkel, of the Department of Zoölogy, University of Chicago, was inadvertently omitted in the April, 1948, issue of *Physiological Zoölogy* (p. 117). It is given in full herewith.

FIG. 2.—Growth of meal-worm larvae at different temperatures and humidities

		Source	Temperature (° C.)	Rel. Hum. (Per Cent)
Upper	*A*	Menusan	31.1	80
	B	Menusan	31.1	50
	C	Present data	30.0	46
Middle	*A*	Menusan	24.7	80
	B	Menusan	24.7	50
	C	Present data	25.1	46
Lower	*A*	Menusan	21.1	80
	B	Menusan	21.1	50
	C	Present data	20.0	64

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

COMPARATIVE PHYSIOLOGY, BY BRADLEY T. SCHEER[1]

Reviewed by RALPH I. SMITH
Department of Zoölogy, University of California, Berkeley

IT IS not easy to evaluate this work, since the subject matter extends over several disciplines, with the result that various readers will judge it from different points of view. This reviewer has tried to eliminate all prejudices except his own, which are those of a general or physiological invertebrate zoölogist; readers with backgrounds in general physiology or biochemistry would undoubtedly see different values in the text. If we consider Dr. Scheer's contribution by attempting to answer certain general questions, the following present themselves: (1) Is the book timely in the sense that it meets a current need? (2) Does the organization of material give a fair and balanced picture of the subject? (3) To what extent may the book be recommended as a classroom text? (4) Does it have value as a general review or monograph over and above its usefulness as a class text? (5) What is its factual accuracy and what are its chief faults? With the comfortable assurance that it is easier to criticize than to write such a book, we may examine the above questions in turn.

At present comparative physiology is rising in importance, partly as a result of the growing appreciation of the need for basic knowledge in almost all fields of science. Weiss (1947) has put forth cogent arguments in his plea for a greater prominence of comparative physiology in the physiological curriculum when he says:

> Let us remember . . . some of the rich returns from past prospecting in invertebrate physiology: the elucidation in one stroke of the working of static sense organs by the smuggling of magnetic otoliths into the crayfish; the giant nerve fibers of earthworms and squids; the tonic muscles of molluscs; the neurospora assay of biochemical systems; the horseshoe-crab model of pacemaker action; the multiple innervation of muscle in crabs, and a host of others. These were strategic break-throughs in an otherwise plodding campaign against the unknown.

Yet, in spite of the case that can be made for the study of comparative and invertebrate physiology, there are no books which adequately present the subject. There are, of course, some standard texts such as Rogers' *Textbook of Comparative Physiology*, in which considerable attention has been given to the physiology of invertebrates; but this text is undocumented and cannot be said to present a picture either of current work in this field or of our knowledge of the physiology of particular groups. Baldwin's absorbing little *Comparative Biochemistry* is unlikely to be excelled as a brief and appealing introduction to a subject, but it purposely avoids detail. Carter's *General Zoology of the Invertebrates* merits distinction as a presentation of the subject from a functional point of view but cannot be considered an advanced text or one adequate for the needs of the comparative biochemist or physiologist. Furthermore, it is not extensively documented, and even at its time of publication (1940) did not cover recent literature. So it must be concluded that Dr. Scheer has produced a book for which there is a present need.

Turning to the question of how well the book covers a subject in need of attention, it is fair to consider the author's aims. From his Preface we learn that he has

> tried to illustrate by excerpts from the recent literature of comparative physiology the state of our knowledge of certain fields and, more important, the potentialities of the comparative method. . . . This book was planned as a text suitable for an advanced university course in comparative physiology. . . . In addition to a possible function as a textbook, however, I hope that this book may interest zoolo-

[1] New York: John Wiley & Sons, Inc.; and London: Chapman & Hall, Ltd., 1948. Pp. x+1–563. $6.00.

gists and physiologists who have had little time to consider the varied and scattered literature outside their immediate fields of research. . . . Instead of the usual treatment by functional units, I have preferred the phylogenetic treatment used in most courses in morphological zoology.

These are worth-while aims. In respect to some, Dr. Scheer has done well. The book is nicely documented, with references given in footnotes. The practice of grouping references on a given topic and of referring to reviews makes the documentation convenient and unobtrusive, without significant detriment to its usefulness. However, in respect to presenting "the potentialities of the comparative method," the author has succeeded less completely. If anything can be said to be the essence of the comparative method, it is to cut across taxonomic lines in the study of processes common in some form to all animals and to trace their modification and evolution. Despite the use of chapter summaries, this book does not, in many cases, achieve the truly comparative synthesis of material that we may see in such reviews as those of Redfield (1933) on the respiratory function of the blood, Yonge (1928 and 1937) on feeding and digestive mechanisms, or Prosser (1946) on invertebrate nervous systems. When, for instance, the reader attempts to resynthesize a comparative picture of feeding and digestive processes, he must proceed from chapter i ("The Physiology of Animals"), which covers very well metabolism and the chemistry of foodstuffs, through seven chapters, each of which reviews the physiology of an invertebrate group, to chapter ix ("Vertebrata: Vegetative Functions"), in which he will be surprised and relieved to find (pp. 369–73) a good summary of feeding methods, chiefly of invertebrates. Again, when we look for a comparative treatment of such subjects as the giant nerve fibers or the respiratory pigments that have arisen in different invertebrate lines, and attempt to find the common needs that have resulted in such structures or substances, we feel that the comparative treatment has fallen short. Other examples could be cited. The facts are there; the synthesis is not.

The above factors bear upon our third question and upon the author's intention of producing a book "suitable for an advanced university course in comparative physiology." Here personal factors and differences between schools may be decisive in the selection of this text for class use. It is this reviewer's belief that the treatment of the subject by taxonomic categories limits the usefulness of the book in courses where lectures and laboratory deal primarily with function, although in advanced courses, where daily reading assignments and other spoon-feeding can be dispensed with, the book might still be of real value, especially since it would have to be used as a general reference rather than as a mirror of the lectures, or vice versa. But for a lecture course without laboratory or one given on an intermediate level, this text may prove unsuitable.

By conceding the advanced nature of the book, we have partly answered our fourth question: Does the work have values other than as a class text? It does. By choice, the author has suppressed a "functional" or broadly comparative approach in favor of a treatment by loose taxonomic groups. The result is, in the author's words, "a series of sketches on the physiology of each of the major animal phyla." In so doing, Dr. Scheer has given his work the character of a treatise on the physiology of invertebrates and has grouped for the general zoölogist a body of information that should infuse a great deal of worth-while physiological material into invertebrate zoölogy courses. It is an unfortunate thing that, while interest in research in invertebrate physiology is growing, too many introductory courses consist largely of an uncomprehending dissection of "types" without much regard for the physiology, functional anatomy, systematics (as opposed to mere "identification"), ecology, and variation among the invertebrates. Dr. Scheer's book should provide for teachers of invertebrate zoölogy source material which cannot but be a refreshing addition to the present fare.

Our last question poses for us the thankless task of evaluating the factual accuracy of the text and of noting its weak points. In respect to much of the material, Dr. Scheer is a better judge than the reviewer and has apparently presented a useful account, particularly in respect to biochemical factors. In general, the task of surveying the extensive and scattered literature seems to have been conscientiously done, and the resulting summaries, while not without interpretations that could be questioned, appear to be of a high order of reliability. In some places one encounters lengthy series of facts that might better be summed up in a generalization; in other places one feels the need for more illustrations. This last is not a serious lack for the zoölogist, but it would seem advisable to include more illustrative material for those students in physiology and biochemistry who may be rela-

tively unfamiliar with invertebrate types and their morphology. And while it is not our purpose to quibble about small omissions and oversights (the search for which in a sense enlivens the reading of the text), it would seem fair to mention that in the effort to close the gap between "old-line" zoölogy and the newer comparative physiology, Dr. Scheer has in some cases oversimplified certain concepts of invertebrate morphology, functional anatomy, and systematics to a point where students may become confused or misled. Thus on page 296 the author fails to recognize that the proximal pigment of the crustacean eye is contained *in* the retinula cells. In the discussion of digestion (p. 536) the statement that "true extra-cellular digestion appears in certain carnivorous gastropods" seems to draw attention from the (elsewhere stated) fact that a true extra-cellular digestion is important in many lower forms, such as flatworms and coelenterates, in which it provides the essential preliminary, though not the final, breakdown of ingested material. At several places in the book some violence is done to the invertebrate coelom. For instance, the statement on page 535 that the coelom is vestigial in most modern representatives of the mollusks, arthropods, echinoderms, and annelids is hardly correct for the last two groups, nor is the statement in the same paragraph that "the coelom of mollusks and arthropods is represented by the pericardium" a fair picture of the situation in the case of the arthropods.

Perhaps it is unfair to stress such points. To most biochemists and physiologists they are unimportant, while by zoölogists they may charitably be overlooked. The author has aimed high enough that some incompleteness is not surprising. Should this book go into a second edition, it is the reviewer's hope that Dr. Scheer will examine the marginal scrawls in copies that have been carefully read by zoölogists, so that such matters may be clarified. To a considerable degree the faults in this work are those attributable to the way in which invertebrate zoölogy is taught today, for in too many cases we have attempted to teach physiology at the expense of a broad general understanding of the lower animals. We tend to teach a static rather than a functional morphology, to stress a knowledge of "types" rather than a fair presentation of variety, and to substitute mere identification for basic systematics. In order for students to gain the full benefits of works such as Dr. Scheer's, we must provide a generally sound basis in the broad aspects of invertebrate zoölogy, lest the present state of imbalance in the field cause us to lose the fruits of the past while still unprepared to advance knowledge of comparative and invertebrate physiology in the future.

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ANIMAL COLOUR CHANGES AND THEIR NEUROHUMOURS[1]

By GEORGE HOWARD PARKER

Reviewed by FRANK A. BROWN, JR.
Northwestern University

THIS is the first comprehensive survey of the field of animal color changes to appear since the extensive reviews of Van Rynberk in 1906 and of Fuchs in 1914. The book brings together all the significant contributions which have been made in the field between 1910 and 1943, a period during which practically all our present detailed understanding of the complex controlling mechanisms involved in the phenomenon has been achieved. Up until about three decades ago it was believed that the chromatic systems of animals were controlled exclusively by nerves. More recently, however, blood-borne agents have been shown also to be of great importance—in fact, in many animals these appear to constitute the sole method of activation of the chromatophores in their normal responses to shade or color of back-

[1] Cambridge, England: At the University Press, 1948. Pp. x+377. $6.50.

grounds. All those hormones and hormone-like agents which influence the chromatophores of animals, together with those chemical factors which many investigators believe to be secreted by nerve terminals to mediate the action of the nerve impulses upon these effectors, are grouped into a single category referred to as "neurohumors." The book, as its full title states, is a review of the subject of animal color changes, especially from the standpoint of an analysis of the neurohumors involved.

The book is divided into ten chapters. Chapter i provides a general introduction. It describes and discusses the types of chromatophores found among animals, the factors normally influencing these effectors, the occurrence of color changes by chromatophore action in the animal kingdom, and, finally, a critical consideration of the principal methods and techniques that have been used to describe chromatophore-induced color changes.

The main body of the volume includes, successively, reviews of the subject of color change and its control in each of the major animal groups in which it occurs, namely, the cephalopods (chap. ii), crustaceans (chap. iii), cyclostomes, elasmobranchs (chap. iv), teleosts (chap. v), amphibians (chap. vi), and reptiles (chap. vii). Chapter viii deals with the development and growth of chromatophores and the establishment of response patterns in developing vertebrate organisms. Approximately the last half of this chapter is devoted to studies of environmental determination of alterations in the quantities of pigments within the chromatophores of adults, or, in other words, morphological color changes. Chapter ix surveys the state of our knowledge concerning the genetics of vertebrate chromatophoral systems.

The last chapter, chapter x, entitled "Special Activities of Vertebrate Chromatophores," contains a discussion of miscellaneous characteristics of behavior of the system. The pulsations of chromatophores which are induced in isolated teleost scales by modification of the ionic content of the medium are treated. There is also included an interesting discussion regarding the resting and active states of vertebrate chromatophores, with a good defense of a stand that the only genuinely active state occurs when the contained pigment is actually in the process of dispersing or of concentrating. According to this concept, a chromatophore may be in a resting state with its pigment in any stage of concentration or dispersion. This view is contrasted with that held by most investigators that only one particular stage of distribution of pigment constitutes the resting one for the given chromatophore type and that any divergence from this stage requires a maintained activity on the part of the chromatophore in response to its controlling agents. In this chapter are also considered the responses of the chromatophore of vertebrates to the temperature and pressure. The major portion of this last chapter comprises an excellent discussion of the functional roles which have been attributed to chromatophore systems, or, as it is entitled, the "Ecology of Colour Changes."

The book is well balanced. The major portion is given over to the vertebrates, as one would expect, in view of the far greater amount of work that has been done upon them. A glance at the Bibliography will quickly reveal that several times as many papers have been published concerning the vertebrates as concerning the invertebrates.

The organization of the subject matter appears for the most part excellent. It is perhaps somewhat misleading to have the topics "Primary Colour Responses" and "Secondary Colour Responses" discussed for the first time in the first chapter on vertebrate color changes. It would seem that a topic of such general application to the color responses of all animals should have been discussed much earlier in the volume, as, for example, in the Introduction, and that it should have included illustrations selected from invertebrate groups as well as vertebrate. It also seems somewhat unfortunate that the last three chapters which contain considerations of many very interesting problems pertaining to chromatophoral systems in general are also treated as though they were exclusively vertebrate ones rather than more general zoölogical ones applicable to all animals showing color changes.

The Bibliography is superb and extremely comprehensive. This section includes about fifty historically important papers published before 1910, about thirty reviews and surveys, and about twelve hundred other papers published from 1910 to 1943 inclusive. One is, furthermore, amazed at the great amount of critical consideration of this vast literature that has been packed into the relatively brief text. Such an intelligible consideration was achievable only by a person possessing the extraordinary grasp of the subject matter and the easy and lucid style of writing which the author of the book has. It is to be regretted, however,

that, as a result of the recent war and of other delays in publication, the literature is surveyed only up to about 1943. For the crustaceans the field is for the most part critically reviewed as of 1937 or 1938. These delays are not quite so serious as might first appear, since research in this field, as in so many others, proceeded at a very low rate during the war years.

The work is well illustrated. As far as this reviewer can judge, the information presented is thoroughly accurate, and the different points of view regarding the major controversial subjects in the field of animal color changes are, in general, quite objectively presented. The book is very stimulatingly written. A wealth of interesting problems awaiting solution are readily apparent to the reader or are specifically called to his attention in every chapter.

In brief, this book is an excellent and welcome addition in a field in which a considerable number of research papers have been published in recent years and over which no adequate comprehensive critical review of this character has appeared. Biologists can now find an easy and pleasant path through a mass of research literature whose contents until now have appeared to many as a discouragingly complex and confusing array of details.